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An Investigation into the Expression and Physiological Function of Mitochondrial Uncoupling Proteins.

A dissertation submitted to Trinity College Dublin
for the degree of Doctor of Philosophy
by
Audrey M. Carroll

Department of Biochemistry
Trinity College Dublin
Dedicated with love to the best Mam and Dad in the World.

"An investigator starts research in a new field with faith, a foggy idea, and a few wild experiments. Eventually the interplay of negative and positive results guides the work. By the time the research is completed, he or she knows how it should have been started and conducted" Donald. J. Cram.

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them" W.L. Bragg.
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Publications
I certify that none of the work in this thesis has been submitted for any degree or diploma at this, or any other, University and that all of the work described in this thesis is entirely my own.

Audrey M. Carroll
DECLARATION

This thesis has not been submitted as an exercise for a degree at any other university. Except where stated, the work described therein was carried out by me alone.

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Summary

Our study provides evidence that rat thymus contains a functioning mitochondrial uncoupling protein (UCP 1). Using reverse transcriptase-polymerase chain reaction (RT-PCR), we showed that rat thymus contains UCP 1 mRNA. Anti-peptide UCP 1 antibodies, that we show to be specific for UCP 1, detected UCP 1 protein in thymus and thymus cell (i.e. thymocyte) mitochondria. We also show that starvation (48-hour) induced no significant change in the abundance of UCP 1 in thymus and thymocyte mitochondria. Evidence for functional UCP 1 in rat thymus mitochondria was obtained using a radiolabeled binding assay in thymus and BAT mitochondria. UCP 1 in thymus mitochondria binds $[^{3}H]$ GDP with similar binding parameters, $B_{\text{MAX}}$ and $K_{D}$ values, as BAT mitochondria. In addition, a large component of the non-phosphorylating oxygen consumption by thymus mitochondria was inhibited by purine nucleotides and activated by nanomolar amounts of fatty-acids, akin to UCP 1 in BAT mitochondria. We also purified UCP 1 from thymus mitochondria by hydroxyapatite chromatography and identified the isolated protein by peptide mass mapping and tandem mass spectrometry using MALDI-TOF and LC-MS/MS, respectively. We conclude that rat thymus contains a functioning UCP 1 protein, which has the capacity to regulate metabolic flux and production and transport of reactive oxygen containing molecules in the thymus.

The study presented here also contributed towards the characterization of a novel peptide UCP 3 antibody, which is sensitive and discriminatory for UCP 3 over UCP 1, UCP 2 and other mitochondrial transporters. We confirm that UCP 3 protein is expressed in rat BAT and skeletal muscle mitochondria. Using the UCP 3 peptide antibody, we confirm and quantify a 2.8-fold increase in UCP 3 expression observed in skeletal muscle mitochondria isolated from fasted rats. We also show that UCP 3 expression in skeletal muscle is temperature-sensitive. We show that UCP 3 expression is increased 1.6-fold in skeletal muscle mitochondria isolated from rats acclimated to the cold (8°C) for 8 weeks, when compared to rats kept at room temperature. We also show that UCP 3 expression is decreased 1.4-fold in skeletal muscle mitochondria isolated from rats acclimated to 30°C for 8 weeks, when compared to rats kept at room temperature. Furthermore, we show a 2.3-fold increase
in UCP 3 expression in skeletal muscle mitochondria isolated from hyperthyroid rats, when compared to euthyroid controls.

Using RT-PCR analysis, we show the novel finding that UCP 3 transcript is expressed in both human thymus and human spleen. Using our anti-UCP 3 peptide antibody, we were able to detect UCP 3 protein in rat thymus and spleen mitochondria. We were also able to detect UCP 3 protein in thymus cells (i.e. thymocytes). Cellular fractionation of the spleen demonstrates that UCP 3 protein is expressed in mitochondria isolated form reticulocytes, monocytes and lymphocytes. Our study also shows the novel finding that starvation induced a 1.5-fold, 3-fold and 1.2-fold increase in UCP 3 expression in thymus, thymocyte and spleen lymphocyte mitochondria, respectively. Starvation had no effect on UCP 3 expression in spleen mitochondria. Therefore, we conclude the novel finding that thymus, thymocyte and spleen mitochondria contain UCP 3 protein and that its expression is starvation sensitive.

UCP 1 is a GDP binding protein and is as such the cause of the high GDP binding capacity of BAT mitochondria. Almost all of the amino acid residues reported to be essential for the binding of GDP by UCP 1 are conserved in UCP 2 and UCP 3, thereby suggesting that UCP 2 and UCP 3 can bind GDP. However, our results show that GDP binding to UCP containing skeletal muscle and spleen mitochondria is of low affinity, with no saturable binding up to 6 µM [3H] GDP. Similarly, it has long been known that purine nucleotides and nanomolar amounts of fatty-acids e.g. palmitate in vitro increase the protonophoric activity of UCP 1 in BAT and from this study, thymus mitochondria. Purine nucleotides and fatty-acids known to inhibit and activate UCP 1’s uncoupling activity had no effect on proton leak in UCP-containing skeletal muscle and kidney mitochondria. Taken together, these results suggest that unlike UCP 1, UCP 2 and UCP 3 don’t seem to display uncoupling activity in isolated mitochondria, thereby inferring that UCP 2 and UCP 3 may function differently to UCP 1.
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Chapter 1

Introduction
Chapter 1
Introduction

[1.1] Mitochondria:

Mitochondria are present in virtually all eukaryotic cells, both plant and animal. Mitochondria vary considerably in size and shape depending on their source and metabolic state. They are typically ellipsoids of ~ 0.5-μm diameter and 1-μm length. An electron micrograph and cross-section of an animal mitochondrion is illustrated in figure 1.1. The mitochondrion is bounded by a smooth outer membrane and contains an extensively invaginated inner membrane. The number of invaginations, called cristae, varies with the respiratory activity of the particular type of cell. The proteins mediating electron transport and oxidative phosphorylation are bound to the mitochondrial inner membrane so that the respiration rate varies with membrane surface area. Enclosed by the inner membrane is the matrix. The matrix contains high concentrations of the soluble enzymes of oxidative metabolism, as well as substrates, nucleotide co-factors and inorganic ions. The matrix also contains mitochondrial genetic machinery, namely DNA, RNA, and ribosomes.

[1.2] Energy Metabolism and Oxidative Phosphorylation:

Metabolism is the overall process through which living systems acquire and utilize the free energy they need to carry out their various functions. In catabolism, energy is released from the stepwise breakdown of foods to carbon dioxide and water. The organism harnesses this energy in a useable form, suitable for driving biological work such as muscle contraction, protein synthesis and ion pumping. The main site of oxygen consumption and carbon dioxide production is the mitochondrion. The mitochondria is also the main site of ATP production. ATP synthesis is coupled to oxygen consumption in mitochondria by a process termed oxidative phosphorylation (reviewed in Nicholls & Ferguson, 1992). Oxidative phosphorylation is the endergonic synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate (Pi) in mitochondria in mammals. This process is best described by the chemiosmotic theory of Mitchell, (1961).
Figure 1.1 (A) Schematic representation of a mitochondrion. (B) an electron micrograph of an animal mitochondrion (www.uwinnipeg.ca/~simmons/cm1503/mitochondria.htm)
In eukaryotic mitochondria, reducing equivalents such as NADH$_2$ and FADH$_2$ derived from the oxidation of reduced carbon compounds (the carbohydrate or fatty acid molecules derived directly from the diet or released from the fuel stores of the body), feed electrons into the electron transport chain (Figure 1.2). The electron transport chain is situated in the mitochondrial inner membrane. Large membrane-bound enzymes, such as glycerol phosphate dehydrogenase, the dehydrogenases of β-oxidation, NADH-Q oxidoreductase (Complex I) or succinate-Q oxidoreductase (Complex II) pass electrons down the gradient of redox potential to the mobile lipid-soluble carrier, ubiquinone (Q). From Q, the electrons pass down through the bc$_1$ complex (Complex III), cytochrome c (a water-soluble, mobile carrier) and cytochrome oxidase (Complex IV) to the final acceptor, oxygen. Coupling of these energy-releasing oxidation reactions to ATP synthesis (i.e. oxidative phosphorylation) is achieved by a chemiosmotic mechanism. As electrons pass down the electron transport chain, Complex I, III and IV translocate protons across the mitochondrial inner membrane from the matrix into the intermembrane space, thus setting up a delocalized trans-membrane proton electrochemical gradient (Δp). This Δp is used for ATP synthesis from matrix ADP and phosphate, by driving protons through the ATP synthase, which is located in the mitochondrial inner membrane and extends into the matrix. This Δp can also drive metabolite transport across the mitochondrial inner membrane. For instance, the mitochondrial inner membrane contains an ADP-ATP translocator that transports ATP out of the matrix in exchange for ADP produced in the cytosol by ATP hydrolysis (Figure 1.3). The ATP generated in the mitochondrial matrix through oxidative phosphorylation is largely utilised in the cytosol to drive such endergonic processes as biosynthesis, active transport and muscle contraction.

[1.3] Proton Leak:

[1.3.1] Natural Proton Leak

Although ATP synthesis is coupled to oxygen consumption via the proton electrochemical gradient Δp, it has long been known from H$^+$/O stoichiometric studies of the electron transport chain and from H$^+$/ATP stoichiometric studies of the ATP synthase, that ATP synthesis is not perfectly coupled to oxygen consumption in
Figure 1.2: An overview of the redox carriers in the mitochondrial respiratory chain.

A diagram of the mitochondrial electron transport chain indicating the pathway of electron transfer (black) and proton pumping (red). Electrons are transferred between Complexes I and III by the membrane-soluble CoQ and between Complexes III and IV by the peripheral membrane protein, cytochrome C. Complex II transfers electrons from succinate to CoQ.
intact mitochondria (Brand et al., 1977). The simplest illustration of this imperfect coupling is the observation that isolated mitochondria still continue to consume oxygen at a low rate when they are prevented from synthesizing ATP by lack of ADP (State 4 respiration) or by the addition of oligomycin (an inhibitor of proton flow through the membrane domain of the ATP synthase). The reason for this discrepancy is that the mitochondrial inner membrane is not absolutely impermeable to protons, i.e. there is an inefficiency in the system, namely a leakage of protons across the mitochondrial inner membrane (Figure 1.3).

Natural proton leak is thought to be a non-enzymic diffusion process that occurs across the inner membrane of all mitochondria and displays a non-linear (non-ohmic) dependence on its driving force, $\Delta p$, such that the leak is maximal when mitochondria are not making ATP (State 4 respiration) and minimal when they are (State 3 respiration) (Nicholls & Rial, 1974; Porter & Andrews, 1998). The mechanism of proton leak is not fully understood. The weight of evidence in the literature suggests that oxygen consumed under these non-phosphorylating conditions is controlled by the inner membrane permeability to protons (Brand, 1990; Brand et al., 1994; Porter & Brand, 1995; Porter, 2001).

Proton leak is not an artefact of isolation as it is found in mitochondria (isolated or in situ) from all tissues and cells studied so far: liver, thymus, lymphocytes, hepatocytes, thymocytes, skeletal muscle, heart and BAT mitochondria (Nicholls & Rial, 1974; Brand et al., 1994; Rolfe & Brand, 1997). Proton leak accounts for approximately 20% of the resting oxygen consumption of cells, and up to approximately 30% of the resting oxygen consumption of rat hindquarter skeletal muscle (Rolfe & Brand, 1997). Clearly proton leak represents a significant inefficiency in oxidative phosphorylation and is a significant contributor to basal metabolism (Porter, 2001).

[1.3.2] Artificially induced proton leak

One of the most notable early successes of the chemiosmotic theory was its explanation of the action of uncoupling agents. Uncoupling agents abolish the link between oxidation and phosphorylation, allowing electron transport to proceed without coupled ATP synthesis (Brand, 2000). Over the years, many compounds,
Figure 1.3: Schematic representation of oxidative phosphorylation in isolated mitochondria.

See text for details.
including 2,4-dinitrophenol (DNP) and carbonylcyanide-\(p\)-trifluoromethoxyphenylhydrazone (FCCP) have been found to “uncouple” oxidative phosphorylation. DNP and FCCP are lipophilic weak acids that can cross the mitochondrial inner membrane in either a protonated or unprotonated state. This sets up a catalytic cycle that dissipates \(\Delta p\) and so allows substrate oxidation to proceed without providing the driving force for coupled ATP synthesis (Brand, 2000) (Figure 1.4). In fact, uncoupling of mitochondria has been used to reduce body fat in humans. The artificial uncoupler 2,4-dinitrophenol (DNP) has been used for this purpose for many years (Parascandola, 1974). DNP was introduced as an anti-obesity drug in the 1930’s and used with considerable success, though reports of side effects (cataracts) and some deaths from overdose led to it being chased off the market by the U.S. Food and Drug Administration (FDA) in 1938.

[1.3.3] Protein-mediated proton leak

In addition to diffusion-mediated natural “proton leak”, it is widely known that the mitochondrial inner membrane of the brown adipocyte contains an uncoupling protein (UCP; originally called thermogenin) that can override the “diffusion mediated or natural proton leak” enabling dissipation of the proton gradient independently of ATP utilization, thus enabling fuel to be oxidised for direct production of heat in thermoregulation (Nicholls & Locke, 1984). Recent research has shown that there is a family of UCP’s occurring in BAT and other tissues, namely UCP 2 and UCP 3. Hence UCP of BAT has been renamed UCP 1 (Fleury et al., 1997). UCP 1, when activated, catalyses rapid proton leak across the mitochondrial inner membrane, leading to physiologically important non-shivering thermogenesis (Nicholls & Locke, 1984) (Figure 1.4).

[1.4] Brown adipose tissue and Uncoupling Protein-1 (UCP 1)

Much non-shivering thermogenesis (also called metabolic thermogenesis) in small mammals is achieved in the brown adipose tissue (BAT) (Ricquier & Bouillaud, 2000a). The thermogenic function of BAT was demonstrated in the early 1960’s when several research groups reported that BAT produces heat, in particular under conditions requiring extra heat production, such as cold-exposure, birth or
Figure 1.4 The chemiosmotic proton circuit across the mitochondrial inner membrane of isolated mitochondria.

The lower circuit, consisting of substrate oxidation (electron transport chain) and the enzymes of ATP production, results in coupled oxidative phosphorylation. Uncoupling of oxidative phosphorylation occurs via substrate oxidation, a natural leak, artificially-induced leak (2,4-DNP & FCCP) and a protein-mediated proton leak pathway (UCP) (Brand, 2000).
arousal from hibernation (Ricquier & Bouillaud, 2000). BAT is a major site of cold- and diet-induced thermogenesis in rodents (DIT) (Rothwell & Stock, 1979). BAT is found in almost all small mammals and in the newborn of larger mammals, such as humans. BAT is found in characteristic deposits scattered in specific areas of the body. The major deposits being interscapular, perirenal, cervical and axillary regions, in the surroundings of the thyroid and thymus, associated with the rib cage and within the thoracic cavity and the abdominal cavity (Néchad, 1986). The topology of BAT is such that, upon activation of brown adipocytes, heat is quickly cleared though large vessels which convey it to the thoracic spinal cord, heart, thoracic structures, brain and kidneys (Ricquier & Bouillaud, 2000). BAT consists of brown adipocytes, which are morphologically and functionally distinct from white adipocytes. Brown adipocytes contain droplets of triglycerides, a central nucleus and numerous mitochondria which are characterized by a highly developed mitochondrial inner membrane (Ricquier & Bouillaud, 2000a).

Given the specific role of BAT in nonshivering thermogenesis, it has always seemed logical that brown adipocytes are equipped with a mechanism that induces uncoupling of respiration in BAT mitochondria. Using photo-affinity labeling experiments, the protein responsible for this uncoupling was identified as thermogenin (now known as UCP 1) (Heaton et al., 1978).

UCP 1, cloned in 1985 (Bouillaud et al., 1985) and called UCP until 1997, is thought to be exclusively expressed in the brown adipocyte (Cannon et al., 1982). UCP 1 is the key molecule in the thermogenic function of brown adipose tissue (BAT). UCP 1 is a 32 kDa protein and is located in the inner membrane of BAT mitochondria, where it functions as both, a proton (H⁺) conductor and chloride ion (Cl⁻) channel (Klingenberg et al., 1990; Huang & Klingenberg, 1996). The thermogenic role of this protein is due to its capacity to dissipate Δρ (Nicholls & Locke, 1984). Consequently, more fuel is oxidised and the liberated energy is dissipated as heat instead of being captured in ATP.

Newborn mammals that lack fur, such as humans, as well as hibernating animals and rodents contain brown adipose tissue (BAT) that contributes to both the maintenance of body temperature in a cold environment through non-shivering thermogenesis and the control of body weight through the regulatory part of diet-
induced thermogenesis (Rothwell & Stock, 1979; Himms-Hagen et al., 1990). It has been shown that during cold acclimation, the capacity of BAT to produce heat is determined by the UCP-1 content of their mitochondria (Hansen & Knudsen, 1986). Active BAT accounts for approximately 50% of heat production in non-shivering thermogenesis in the cold acclimated rat and accounts up to 1% of the body mass of a rat (Foster & Frydman, 1979; Foster 1986).

[1.5] Biochemistry of UCP 1

[1.5.1] Structure:

UCP 1 has been successfully purified from BAT mitochondria (Lin & Klingenberg, 1980). The primary structure of UCP 1 has also been elucidated by N-terminal sequencing (Aquila et al., 1985).

UCP-1 is a 32 kDa monomer of 306 amino acids, the functional unit maybe a homodimer. UCP-1 is proposed to contain six transmembrane helices with both C- and N- termini protruding to the cytosolic side (Figure 1.5) (Klingenberg, et al., 1999a). Its amino acid sequence is highly homologous to that of several ubiquitous mitochondrial inner-membrane carriers, including the ADP/ATP carrier, the phosphate carrier and the oxoglutarate carrier (Klaus et al., 1991). As a member of the mitochondrial carrier family, the structure can be divided into three similar repeat domains of about 100 residues, each containing two transmembrane helices (Palou et al., 1998). Within each domain, two helices are separated on the matrix side by an approx 40 residues long hydrophilic stretch. UCP 1 from hamster contains 28 positively and 19 negatively charged residues, resulting in excess of nine positive charges (Klingenberg et al., 1999a). Most of the charges are localized in the hydrophilic matrix region. Many charges occur at conserved repeats in each domain. The D27 is located at the same position of a lysine characteristic for all ADP/ATP carriers. In the third domain, the first helix is limited by His (H214) instead of Lys, which is unique for UCP. In the matrix region, a well conserved motif KXR is found in all three domains, which is characteristic for the mitochondrial carrier family. Other charged residues are partially conserved in this highly charged region. The His pair HLH (145-147) is found only in UCP 1 (Klingenberg et al., 1999a). The overall
The protein (306 amino acids) has been proposed to consist of three symmetrical membrane-spanning regions each comprising of about 100 amino acids and to contain six transmembrane alpha-helices.
protein structure consists of about 50% α-helix, 30% β-structure, 15% β-turns and 7% random (Palou et al., 1998).

[1.5.2] Synthesis and degradation of UCP-1:

Thermogenesis in BAT is activated in response to cold exposure, chronic overeating, free fatty acids and β3-adrenergic receptors (Ricquier et al., 1986). The concentration of fatty acids in BAT is controlled by the hormone norepinephrine (noradrenaline).

Norepinephrine plays an important role in brown adipocytes, promoting UCP1 activity (UCP activation) and differentiation (UCP synthesis) (Cannon et al., 1996). The major adrenoreceptor mediating the effects of norepinephrine in mature BAT cells is β3-adrenoreceptor, which is mainly expressed in white and brown adipocytes.

Upon cold-exposure, norepinephrine is released from sympathetic terminals and binds to its cell-surface receptor (Figure 1.6). The norepinephrine-receptor complex stimulates adenylate cyclase to synthesise cAMP, thereby causing cAMP levels to rise. cAMP binding allosterically activates cAMP-dependent protein kinase (cAPK). cAPK phosphorylates hormone-sensitive triacylglycerol lipase, thereby activating thermogenesis. Finally, the activated lipase hydrolyzes triacylglycerols to yield the free fatty acids that open the proton channel. Free fatty acids overcome the purine nucleotide (GDP) block of the proton channel formed by the uncoupling protein, allowing protons (H+) to enter the mitochondrion uncoupled from ATP synthesis (Figure 1.6).

Another key downstream target of cAPK is CREB (cAMP-response-element-binding-protein) which stimulates transcription from the UCP gene (Silva & Rabelo, 1997). The UCP gene is mainly regulated at the transcriptional level (Figure 1.7). Besides noradrenaline (NE), another activator of UCP transcription is triiodothyronine (T3), which acts in connection with NE, since NE stimulation markedly increases thyroxine deiodinase activity of brown adipocytes, thus allowing high intracellular generation of T3 from thyroxine. T3 stimulates UCP gene expression via thyroid hormone response elements (TRE’s) (Silva & Rabelo, 1997). Retinoic acid, the natural active form of vitamin A, has been shown to stimulate
Figure 1.6: The mechanism of hormonally-induced uncoupling of oxidative phosphorylation in brown fat mitochondria.
Figure 1.7: The mechanism of hormonally-induced activation of UCP 1 in brown adipocytes.
UCP gene transcription (Cassard-Doulcier et al., 1994; Larose et al., 1996). Retinoic acid and vitamin D receptors bind to the specific response elements in the genes as heterodimers with other receptors, most commonly with the so-called RXR receptors. It is also known that activators of peroxisome proliferation activating receptors (PPAR), such as pioglitazone activate UCP 1 gene expression (Sears et al., 1996). Sears et al. (1996) have shown that PPARγ-RXR heterodimers are necessary for cAMP-mediated stimulation of the UCP gene.

Concerning UCP degradation, it has been suggested that a post-translational regulatory mechanism exists which ensures the rapid degradation of newly synthesized molecules when the physiological stimulation ceases (Puigserver et al., 1992; Bonet et al., 1995).

[1.6] UCP homologues

Recent research has shown that there is a family of UCP’s occurring in BAT and other tissues (Fluery et al., 1997; Boss et al., 1997; Vidal-Puig et al., 1997).

UCP-2 was cloned using primers to UCP-1 (Fluery et al., 1997). UCP 2 is a 33 kDa protein and shares 59% homology with UCP 1. UCP 2 is 308 amino acids in length with a pI of 9.7-9.8. UCP 2 mRNA is found widespread in mammalian tissues of mice, rat and humans with highest levels being found in spleen, thymus, intestine, lung, brain, kidney, white adipose tissue (WAT), BAT, foetal liver, in the Kupffer cells of the mature liver, myocytes, lymphocytes, macrophages and in the female reproductive tract (Fluery et al., 1997; Gimeno et al., 1997; Larrouy et al., 1997; Nègre-Salvayre et al., 1997; Gong et al., 1997; Boss et al., 1998; Hodný et al., 1998; Ricquier & Bouillaud, 2000; Pecqueur et al., 2001; Pedraza et al., 2001; Krauss et al., 2002; Horvath et al., 2003; Rousset et al., 2003). On the other hand, UCP 2 protein is only found in mouse and rat spleen, lung, stomach, kidney and thymus (Ricquier & Bouillaud, 2000; Pecqueur et al., 2001; Krauss et al., 2002; Echtay et al., 2001a).

UCP 3 was also cloned (Boss et al., 1997; Vidal-Puig et al., 1997). UCP 3 has two isoforms; a long form (UCP 3L) and a short form (UCP 3S) (Boss et al., 1997; Vidal-Puig et al., 1997). The short form has 275 amino acid residues whereas the long form has 312 amino acid residues. The two human isoforms have amino
acid homologies of 57% and 73% to UCP-1 and UCP-2 respectively. UCP-3s lacks the sixth potential trans-membrane region and putative nucleotide binding site. Expression of UCP-3 mRNA has only ever been detected in BAT and skeletal muscle with transcript having particular prominence in tensor fascia latae (fast-twitch, glycolytic), tibalis anterior (fast-twitch, oxidative/glycolytic) and gastrocnemius (mixed) muscles and lower levels in the soleus muscle (slow-twitch, oxidative) (Boss et al., 1997 & 1998; Vidal-Puig et al., 1997; Cadenas et al., 1999; Giacobino et al., 1999; Pedraza et al., 2001). UCP 3 protein has only ever been detected in mouse and rat skeletal muscle (Jeżek et al., 1999; Gong et al., 2000; Li et al., 2000; Zhou et al., 2000; Vidal-Puig et al., 2000 & 2000a; Cadenas et al., 2002; Harper et al., 2002; Cunningham et al., 2003; Hesselink et al., 2003) and BAT mitochondria (Jeżek et al., 1999; Gong et al., 2000; Li et al., 2000; Harper et al., 2002; Cunningham et al., 2003).

More recently, a fourth UCP (UCP 4) has been identified (Mao et al., 1999). UCP 4 is a 36 kDa protein of 323 amino acids. UCP 4 is 29 %, 33 % and 34 % homologous to UCP 1, UCP 2 and UCP 3 respectively. UCP 4 transcripts are exclusively expressed in both fetal and adult brain tissues.

A fifth UCP (UCP 5) has also been identified (Yu et al., 2000). UCP 5 is also termed brain-specific mitochondrial carrier protein or BMCP-1. UCP 5 mRNA expression is widespread in the brain, with some expression in testis, heart, kidney, and uterus (Yu et al., 2000). UCP 5 isoforms have been discovered; a short form (UCP 5 S), a short form with insert (UCP 5 SI), and a long form (UCP 5 L).

Vercesi et al. (1995) discovered a plant uncoupling protein (PUMP) in potato tubers. Laloi et al. (1997) reported the cloning of another plant UCP, present in Solanum tuberosum and referred to as StUCP. cDNA's of a UCP homologue present in Arabidopsis thaliana were also cloned and referred to as AtUCP or AtPUMP (Maia et al., 1998). Costa et al. (1999) identified PUMP in tomatoes. Interestingly, using antibodies against plant UCP, Jarmuszkiewicz et al. (1999) detected a mitochondrial uncoupling protein in the non-photosynthetic soil amoeboid protozoan Acanthamoeba catellanii. Similarly, Jarmuszkiewicz et al. (2000) identified an uncoupling protein in mitochondria from Candida parapsilosis (CpUCP), a non-fermentative parasitic yeast. Recently, a new protein named avUCP, which shares a
70% amino acid identity with both UCP 2 and UCP 3, was identified in chicken skeletal muscle (Raimbult et al., 2001).

Despite the existence of several UCP homologues, the study presented here only sought to investigate the expression and physiological function of UCP 1, UCP 2 and UCP 3.

[1.7] UCP mRNA expression

Northern blot analysis, using radiolabelled nucleotides, has been key in identifying patterns of mRNA expression for UCP 1, UCP 2 and UCP 3 (Boss et al., 1997; Gong et al., 1997; Larrouy et al., 1997; Masaki et al., 1997; Nègre-Salvayre et al., 1997; Boss et al., 1998; Hodný et al., 1998; Yoshitomi et al., 1998; Giacobino et al., 1999; Matthias et al., 1999; Richard et al., 1999; Ricquier & Bouillaud, 2000; Pecqueur et al., 2001; Pedraza et al., 2001; Krauss et al., 2002; Horvath et al., 2003). Northern blot analysis has shown that UCP 1 mRNA is expressed in brown adipose tissue (BAT), where it provides the basis for nonshivering thermogenesis in mammals (Cannon et al., 1982; Ricquier & Bouillaud, 1986; Boss et al., 1997; Nègre-Salvayre et al., 1997; Boss et al., 1998; Giacobino et al., 1999; Richard et al., 1999; Boss et al., 2000; Ricquier & Bouillaud, 2000; Pedraza et al., 2001). As mentioned earlier (section 1.6), Northern blot analysis has also shown that UCP 2 mRNA is present in a panoply of tissues of mice, rat and humans with highest levels being found in spleen, thymus, intestine, lung, brain, kidney, white adipose tissue (WAT), BAT, foetal liver and in the Kupffer cells of the mature liver (Fluery et al., 1997; Larrouy et al., 1997; Nègre-Salvayre et al., 1997; Gong et al., 1997; Boss et al., 1998; Hodný et al., 1998; Ricquier & Bouillaud, 2000; Pecqueur et al., 2001; Pedraza et al., 2001; Krauss et al., 2002; Horvath et al., 2003). Similarly, Northern blot analysis shows that UCP 3 transcript has only ever been detected in BAT and skeletal muscle with transcript having particular prominence in tensor fascia latae (fast-twitch, glycolytic), tibialis anterior (fast-twitch, oxidative/glycolytic) and gastrocnemius (mixed) muscles and lower levels in the soleus muscle (slow-twitch, oxidative) (Boss et al., 1997 & 1998; Vidal-Puig et al., 1997; Cadenas et al., 1999; Giacobino et al., 1999; Pedraza et al., 2001).
The amplification of the polymerase chain reaction (PCR) technique provides another method of mRNA analysis for UCP’s (Saiki et al., 1985). Reverse-transcriptase polymerase chain reaction (RT-PCR) has been very successful in detecting changes in UCP mRNA expression. Using RT-PCR analysis, Oberkofler et al. (1997) showed that UCP 1 mRNA is expressed in adipose tissue of obese and non-obese humans. Larrouy et al. (1997) demonstrated that UCP 1 mRNA was not detected in mature liver but showed that UCP 2 mRNA was expressed preferentially in the Kupffer cells of the mature liver. Cabrero et al. (1999) showed that etomoxir induced a 3.6-fold increase in UCP 3, but not UCP 2 mRNA levels in a primary culture of rat preadipocytes. Similarly, Cabrero et al. (2000 & 2001) showed that UCP 2 and UCP 3 mRNA levels were down-regulated by thiazolidinediones in C2C12 myotubes, whereas UCP 3 mRNA levels were upregulated in C2C12 myotubes after etomoxir treatment. With the knowledge that RT-PCR is 1,000-10,000 fold more sensitive than the traditional Northern blotting techniques in detecting gene expression (He et al., 1995) and the fact that RT-PCR has previously been successful in detecting changes in UCP expression, one of the aims of this study was to apply RT-PCR to tissues, previously investigated with Northern blotting to help elucidate further locations and possible physiological function(s) of UCP’s.

[1.8] UCP protein expression

UCP 1 protein from rat, hamster, guinea-pig, rabbit or mouse, is recognized by anti-rat-uncoupling protein antiserum (Ricquier & Bouillaud, 1986). Up to recently, UCP 1 has only ever been associated with BAT mitochondria. No significant quantities of UCP 1 were detected in immunological studies of liver, heart, epididymal white fat, parametrial white fat or thigh muscle-even from cold acclimated animals (Ricquier & Bouillaud, 1986). A study by Nibbelink et al. (2001) showed that UCP 1 is expressed in uterine longitudinal smooth muscle cells, but this finding has since been disproven (Rousset et al., 2003). Commercial and in-house anti-UCP 1 peptide antibodies have been successful in determining factors affecting UCP 1’s protein expression. It has been shown that cold-acclimation induces an increase in UCP 1 protein expression in BAT mitochondria isolated from rats (Desautels et al., 1978; Heaton et al., 1978; Ricquier et al., 1979; Swick & Swick,
1986; Sundin et al., 1987; Klaus et al., 1991; Nicholls & Rial, 1999; Nedergaard et al., 2001). By contrast, starvation has been shown to decrease UCP 1 protein expression in BAT mitochondria (Rothwell et al., 1984; Trayhurn et al., 1988; Boss et al., 1998b; Nedergaard et al., 2001). In addition, certain dietary regimes such as cafeteria diets, high-fat diets and high-sucrose diets have been shown to increase UCP 1 protein content in BAT (Himms-Hagen, 1986; Nedergaard et al., 2001).

Lipopolysaccharide (LPS) and starvation has been shown to increase UCP 2 protein expression in lung and stomach (Pecqueur et al., 2001). Starvation has been shown to increase UCP 3 protein expression levels in mouse and rat skeletal muscle mitochondria (Cadenas et al., 1999; Sivitz et al., 1999; Moreno et al., 2003). Short-term cold exposure has also been shown to increase UCP 3 protein expression in rat skeletal muscle mitochondria (Simonyan et al., 2001; Wang et al., 2003). Administration of thyroid hormone to mice and rats has been shown to increase UCP 3 protein expression in skeletal muscle mitochondria (Lanni et al., 1999 & 2003; Lombardi et al., 2002; Moreno et al., 2003). High-fat diets increased UCP 3 protein expression by approximately 40%, compared to low fat diets in skeletal muscle mitochondria (Hesselink et al., 2003). Refeeding on a high fat diet results in a 2-fold increase in UCP 3 protein expression (Crescenzo et al., 2003). Hypoxia and exercise induced a 4 to 6-fold increase in UCP 3 protein expression in rat skeletal muscle (Zhou et al., 2000).

To date, commercial UCP 3 peptide antibodies are available from Calbiochem, Chemicon International, Lilly and Alpha-diagnostics. Similarly, UCP 3 peptide antibodies have been developed in-house by several research groups (Vidal-Puig et al., 2000; Hesselink et al., 2003). However, the sensitivity and discriminatory nature of these commercial and in-house antibodies to each other and to other members of the mitochondrial transporter family have yet to be adequately demonstrated. With all this in mind, we designed a UCP 3 peptide antibody, (see section 2.18, Chapter 2), which we show to be sensitive and discriminatory for UCP 3 over UCP 1 and UCP 2 and other mitochondrial transporters (Cunningham et al., 2003). Therefore, one of the aims of this study was to use our UCP 3 antibody to investigate changes in UCP 3 protein expression under various physiological conditions.
Purine nucleotide binding to UCP's

Binding of nucleotides is the best defined biochemical function of UCP 1 (Klingenberg & Echtay, 2001). Nucleotide binding by UCP 1 has been a key in identifying and isolating this proton transporting protein (Heaton et al., 1978; Lin & Klingenberg, 1982). Nucleotides bind only from the cytosolic side of UCP 1 (Klingenberg & Huang, 1999). UCP 1 accepts with strong preference the purine ribose di- and tri-nucleotides: GDP, GTP, ATP and ADP, whereas the monophosphates GMP and AMP are poor ligands (Klingenberg, 1988). UCP 1 only accepts the free forms of nucleotides, not the Mg\(^{2+}\) complexes (Klingenberg, 1988; Huang & Klingenberg, 1995a). UCP 1 is predicted to behave as a dimer in the mitochondrial inner membrane (Klingenberg, 1984). Furthermore, it has been shown that only one nucleotide binds per UCP 1 dimer (Klingenberg & Huang, 1999). Due to the presence of UCP 1, functioning brown adipose tissue mitochondria (BAT) are able to adapt to their thermogenic capacity in response to both environmental temperature and dietary signals (Peachey et al., 1988). Rafeal and Heldt, (1976), found that purine nucleotide binding capacity varied with the thermogenic capacity of BAT. Radiolabeled and fluorescent-labeled purine nucleotide binding assays are the most sensitive and specific methods for determining the presence of UCP 1 (Milner et al., 1988). The specific binding of radiolabelled GDP has been used extensively as an indication of the thermogenic capacity of BAT (Sundin & Cannon; 1980; Nedergaard & Cannon, 1985; Sundin et al., 1987).

One of the most remarkable features of nucleotide binding to UCP 1 is its strong pH dependence (Klingenberg & Huang, 1999). The affinity for purine and pyrimidine nucleotides decreases upon increasing pH, notably above 7.2 (Klingenberg & Huang, 1999). Site-directed mutagenesis studies with the help of a highly specific Woodward reagent K, identified a glutamic acid residue at position 190 (E190N) within the fourth helix, to be essential for the pH sensitivity of nucleotide binding to UCP 1 (Echtay et al., 1997) (Figure 1.8). Echtay et al. (1997) also showed that mutational neutralization in E190N largely abolished the pH dependence of nucleotide binding and increased the affinity at pH >7. Mutation of H214 within helix 5 and possibly quite close to E190 decreased pH dependence of binding only for NTP but not for NDP (Echtay et al., 1998). The kinetics of
Figure 1.8: Localisation of the mutagenized residues (as discussed in text) in hamster UCP 1. In all cases the charged residues were replaced by neutral residues.
nucleotide binding to UCP 1 from BAT mitochondria, previously determined by Huang et al. (1998), shows fast and slow phases of binding to UCP 1.

It has been observed in some laboratories that the binding capacity of UCP 1 varied with short-term changes of temperature acclimation (Nedergaard & Cannon, 1985; Trayhurn et al., 1987). On the transfer of rats from 27°C to 4°C for only 20 minutes, GDP binding increased 1.3-fold (Swick & Swick, 1986). Reversibly, on short-term warm adaptation, the binding was masked. Since no de novo synthesis occurs in this short time, the results suggested an "unmasking" of binding sites (Swick & Swick, 1986; Klingenberg & Echtay, 2001). The masking phenomenon relates primarily to changes in the number of nucleotide-binding sites after the animal undergoes acute temperature changes i.e. before protein synthesis takes place (Desautels et al., 1978; Nedergaard & Cannon, 1985; Gribskov et al., 1986; Peachey et al., 1988; Huang & Klingenberg, 1995). It is known that the UCP 1 purine nucleotide binding site in BAT mitochondria isolated from rats or hamsters kept at room-temperature or warm acclimated is occupied by endogenous ATP and are therefore masked (Klingenberg & Huang, 1999). In isolated mitochondria, UCP 1 can be completely unmasked by treatment with Dowex (21K, anion exchanger) at high pH.

A mechanism for masking/unmasking phenomenon incorporating the ATP hypothesis is illustrated in figure 1.9 (adapted by Klingenberg & Huang, 1999). It is thought that at thermoneutrality (28°C), BAT mitochondria are largely coupled since UCP 1 activity is chiefly inhibited by endogenous ATP. The basal free fatty-acid concentrations are low. A low basal thermogenic activity is maintained by the small number of free UCP 1 molecules. When animals are acutely cold-adapted (4°C) or noradrenaline stimulated, fatty-acids are released which increase proton transport activity through UCP 1 and deplete endogenous ATP by fatty-acid activation, where ATP is converted to AMP. This acute drop in endogenous ATP concentration results in dissociation of prebound ATP from UCP 1, yielding more free UCP 1 molecules and thereby unmasking. The high fatty-acid and low ATP concentrations allow a high thermogenic activity (Huang & Klingenberg, 1995). In isolated mitochondria, UCP 1 could be completely unmasked by treatment with Dowex at a high pH.
**Figure 1.9: Proposed mechanism for the masking/unmasking phenomenon**

(as adapted by Klingenberg & Huang, 1999)
Dowex (21K) is an anion exchanger that removes endogenous residual bound nucleotides from isolated mitochondria.

With the discovery of a family of UCP’s i.e. UCP 2 and UCP 3 (Fluery et al., 1997; Boss et al., 1997), our study sought to investigate whether mitochondria constitutively expressing UCP 2 and UCP 3 can bind purine nucleotides, akin to UCP 1. There is some evidence to support the hypothesis that UCP 2 and UCP 3 can bind purine nucleotides. Based on the relatively high sequence homology of UCP 2 and UCP 3 to UCP 1, these new UCP homologues were predicted to bind GDP. Some of the amino acids known to be involved in purine nucleotide binding in UCP 1 have been identified from chemical modification and site-directed mutagenesis studies (Klaus et al., 1991; Klingenberg & Huang, 1999). The amino acids reported to be involved in purine nucleotide binding in studies on mouse UCP 1 are 75-80% conserved in UCP 2 and the long form of UCP 3 (UCP 3L) (Klaus et al., 1991; Klingenberg & Huang, 1999) (Figure 1.10). Chemical modification studies have shown the contiguous sequence EGPAAFFKG to be involved in purine nucleotide binding in mouse UCP 1 (Klaus et al., 1991). Similar contiguous sequences of EGPRAFYKG and EGPTAFYKG occur at equivalent positions in UCP 2 and UCP 3 respectively. In addition, site-directed mutagenesis studies on Arg 83, Arg 182, Arg 276 (helix 6) of mouse UCP 1, have shown them to be essential for purine nucleotide binding (Modriansky et al., 1997; Klingenberg & Huang, 1999; Porter, 2001). Equivalent arginines exist in equivalent positions in UCP 2 and the long form of UCP 3L. A comparison of rat UCP 1 with mouse UCP 1 reveals that the consensus sequence (EGPAAFFKG) and equivalent arginines (Arg 84, Arg 183, Arg 277) are present in rat UCP 1. The consensus sequence and equivalently positioned arginines are present in rat UCP 2 and rat UCP 3 (Figure 1.10). Therefore, there is certainly circumstantial evidence from primary sequence studies that UCP-2 and UCP-3 can bind purine nucleotides.

Other evidence showing that UCP 2 and UCP 3 can bind purine nucleotides have come from heterologous yeast and E.coli expression systems. Zackova et al. (2003) has shown that E.coli or yeast expressing UCP 2 and UCP 3 exhibit high affinity [³H] GTP binding, similar to UCP 1. Jekabsons et al. (2002) has also shown that recombinant human UCP 2, ectopically expressed and solubilised in bacterial
The diagram shows the consensus sequence \textit{EGPAAFFKG} in UCP 1 and its equivalent \textit{EGPRAFYKG} in UCP 2 and \textit{EGPTAFYKG} in UCP 3. Also shown are Arg 277 of rat UCP 1, equivalently positioned Arg 279 of rat UCP 2 and Arg 278 of rat UCP 3. Both the contiguous sequence of EGPAAFFKG and the Arg 276 have been implicated in purine nucleotide binding.
inclusion bodies bind purine and pyrimidine nucleoside triphosphates with low micromolar affinity, thus providing more evidence that UCP 2 and UCP 3 may bind purine nucleotides. However, data showing purine nucleotide binding to isolated mitochondria containing UCP 2 and UCP 3 has been severely lacking. Therefore, one of the aims of this study was to investigate whether mitochondria containing UCP 2 and UCP 3 can bind purine nucleotides, akin to UCP 1 in BAT mitochondria.

[1.10] Effects of fatty-acids and purine nucleotides on UCP function

UCP 1’s mediated uncoupling of oxidative phosphorylation is inhibited by purine nucleotides and activated by nanomolar amounts of free fatty-acids and by long chain fatty-acyl CoA esters (Nicholls & Rial, 1974). Medium to long chain fatty-acids induce UCP 1’s uncoupling activity in BAT mitochondria (Klingenberg & Huang, 1999). Fatty-acids such as palmitic, oleic, linoleic and linolic fatty-acids are good activators of UCP 1 induced proton transport (Klingenberg & Echtay, 2001a). An oligoisoprenoid acid such as retinoic acid is a good activator of UCP 1 uncoupling activity in BAT mitochondria and yeast mitochondria expressing UCP 1 (Rial et al., 1999). Although the inhibition by nucleotides is well established, the mechanism by which fatty-acids activate UCP 1 remains controversial.

The mechanism of uncoupling by UCP 1 has devolved into two models; the fatty-acid protonophore model and the proton buffering model. The fatty-acid protonophore model, illustrated in figure 1.11 (A), was introduced by Garlid et al. (1996). According to this model, UCP catalyses flip-flop of the anionic head group of fatty-acid from the mitochondrial matrix to the outer leaflet of the mitochondrial inner membrane. Transport of the anion is driven by the high, inside-negative membrane potential (∆Ψ). After the carboxyl head group has crossed the membrane, it picks up a proton, and the protonated fatty-acid spontaneously and rapidly flip-flops back to the matrix side where deprotonation completes the cycle. The net result of the cycle is delivery of protons with charge to the matrix. Thus, fatty-acids behave as cycling protonophores. According to this model, UCP does not conduct protons at all but transports fatty-acid anions. Proton flux occurs independently by non-ionic diffusion (Garlid et al., 1998).
Figure 1.11: Proposed mechanisms by which free fatty-acids activate $H^+$ transport by UCP 1.

(A) Fatty-acid protonophore model, proposed by Garlid, where fatty-acids act as cycling protonophores. Fatty-acid anions are transported out from the matrix and protonated fatty-acids return into the mitochondrial matrix by flip flop. The $H^+$ is then released in the mitochondrial matrix. (B) Proton buffering model, proposed by Klingenberg, where fatty-acid carboxyl groups acting as $H^+$ buffers in conjunction with resident $H^+$ buffering amino acids in the translocation channel.
In the **proton buffering model**, protons are directly transported by UCP. The proton buffering model is illustrated in figure 1.11 (B) and was introduced by Winkler and Klingenberg, (1994). According to this model, intramembrane fatty-acids insert their head groups into the proton transport pathway and provide buffering sites to assist proton translocation through UCP 1. UCP 1 actually transports protons and fatty-acids act as buffering co-factors that operate in conjunction with resident proton-conducting amino acids, such as histidines (Biengraeber *et al.*, 1998; Winkler & Klingenberg, 1994).

There is a lot of evidence to support and contradict both models. Evidence favouring the fatty-acid protonophore model comes from the discovery that alkylsulfonates, ranging in alkyl chain length between $C_{11}$ and $C_{16}$, are transported by UCP 1 (Ježek *et al.*, 1990 & 1994; Garlid *et al.*, 2001). Alkylsulfonates can be regarded as “permanent” fatty-acid anion analogs, since at physiological pH they cannot pick up a $H^+$. Alkylsulfonates are competitive inhibitors of UCP-mediated Cl⁻ transport (Garlid *et al.*, 2001). Ježek *et al.* (1994) tried to derive the alkylsulfonate transport activity by measuring the competition with Cl⁻ uptake. Only with the longer chain $C_{11}$-sulfonate the competitive suppression of the lauric acid-catalyzed $H^+$ transport was demonstrated. A major problem with these experiments is the sensitivity towards GDP. With increasing chain length the uptake of alkylsulfonate ($C_6$ and $C_{11}$) becomes more insensitive to GDP (Ježek *et al.*, 1994). Since this model requires that the protonated acid permeates across the lipid bilayer, Ježek *et al.* (1994) tested the model by comparing the activation of UCP 1 by a number of organic acids with the ability to permeate across the bilayer. These authors found that the bulky-planar structure of the benzene ring prevented the flip-flop of compounds like the phenylhexanoic acid. However the demonstration that all-\textit{trans} retinoic acid is a powerful activator of UCP 1 seems to violate this hypothesis (Rial *et al.*, 1999). Furthermore, Klingenberg & Huang (1999) have found a fatty-acid derivative, \(\omega\)-glucopyranoside palmitic acid that should not permeate the bilayer but does activate UCP 1.

There is a lot of evidence to support the fatty-acid protonophore model. In this model, the carboxylate would bind protons and deliver them inside UCP 1 from which translocation would take place. As with purine nucleotide binding to UCP 1
(section 1.9), one of the first striking features of the fatty-acid activation of UCP 1 is its pH dependency. There is a sharp increase in proton conductance over the pH range 7.0-8.0 (Nicholls, 1974). The pH dependency may be an indication of the involvement of an amino acid side chain in UCP 1. Klingenberg & Huang, (1999) have shown that His 145 and His 147 were required for fatty-acid dependent proton transport while anion transport was unaffected when these residues were mutated (Figure 1.8). Klingenberg & Huang, (1999) proposed that these residues, which are located on the matrix side of the protein, could be the final acceptors in the proton translocation chain. Biengraeber et al. (1998) has also reported the involvement of Asp 27 in proton but not chloride transport (Figure 1.8).

A final question should also be considered. The fatty-acid cycling hypothesis proposes that the UCP’s are carriers for the fatty-acid anion hence in UCP 1, there would be no transport activity in their absence (Garlid et al., 1998). This argument contrasts with the earlier observations of the bioenergetic properties of BAT mitochondria that signified, even upon removal of all traces of endogenous fatty-acids, mitochondria remained uncoupled (Ricquier & Bouillaud, 1986). Rial & González-Barroso (2001) would argue that fatty-acids are activators of proton transport by UCP 1 but they are not an absolute requirement for UCP 1 function.

Experimentally, the fact that UCP 1’s uncoupling can be “turned on” by fatty-acids and “turned off” by purine nucleotides allows a distinction to be made between UCP 1 proton conductance pathway and other pathways of mitochondrial proton conductance. This purine nucleotide inhibition and fatty-acid activation of UCP 1 activity in BAT mitochondria has become an interesting paradigm for the study of UCP 2 and UCP 3.

To investigate whether fatty-acids activate UCP 2 and UCP 3’s function, in a similar manner to UCP 1, it was necessary to assume that UCP 2 and UCP 3 are natural uncouplers. There are several lines of evidence to support the hypothesis that UCP 2 and UCP 3 are natural uncouplers. As stated earlier, UCP 2 and UCP 3 are 59% and 56% homologous to UCP 1 respectively and 71% identical to each other (Lowell, 1999). UCP 2 and UCP 3 are much less homologous to other members of the mitochondrial carrier superfamily (Lowell, 1999). Based upon the high homology of UCP 2 and UCP 3 with UCP 1, compared to its lower homology with
other members of the mitochondrial carrier family, it is predicted that UCP 2 and UCP 3 share biochemical functions with UCP 1, including uncoupling activity. The UCP 3 gene is found adjacent to that for UCP 2 on chromosome 7 in mice and on chromosome 11 in humans (Harper et al., 2001; Jabur et al., 1999). These chromosomes have been linked to hyperinsulinemia and obesity, and it is hypothesized that UCP 2 and UCP 3 are the peripheral target for energy dissipation in the regulation of body weight (Jabur et al., 1999). Most of the evidence to date that shows UCP 2 and UCP 3 are uncoupling proteins came from studies using heterologous yeast expression systems, UCP's reconstituted into proteliposomes, transgenic overexpression systems and gene knockout studies.

[1.10.1] Heterologous yeast expression systems

Direct studies of the function of the UCP 1 homologues have been done most often using heterologous yeast and mammalian cell expression systems. Expression of UCP 2 and UCP 3 in *S. cerevisiae* appears to confirm their uncoupling activities: membrane potential is lowered and state 4 oxygen consumption rates are increased (Echtay et al., 1999; Giacobino et al., 1999; Hagen et al., 1999; Hinz et al., 1999; Hinz et al., 1999a; Jabur et al., 1999). Measurements of mitochondrial State 4 respiration, *i.e.* respiration in the absence of ADP, which is primarily due to proton leak, shows a 50% increase in mitochondria from yeast expressing UCP 2, compared with that of the control (Fluery et al., 1997). Similarly, UCP 3 has been shown to increase whole yeast basal oxygen consumption (Hinz et al., 1999; Zhang et al., 1999). The increase in whole yeast basal oxygen consumption with UCP 3 was even greater than that observed for UCP 1 (Zhang et al., 1999). In isolated mitochondria, however, the increase in State 4 respiration with UCP 3 (35%) was small compared to the increase with UCP 1 (100%). However, the increase in state 4 respiration of yeast mitochondria expressing UCP 2 and UCP 3 was insensitive to 1 mM purine nucleoside di- and tri-phosphates (Hagen et al., 1999; Rial et al., 1999; Zhang et al., 1999; Hagen et al., 2000). These observations suggest that UCP 2 and UCP 3 exists in a “deranged” confirmation and does not obtain native function in yeast expression systems (Hinz et al., 1999; Heidkaemper et al., 2000; Klingenberg & Echtay, 2001a; Harper et al., 2002). Other principal concerns with overexpressing a heterologous
protein in yeast have been noted in the literature (Stuart et al., 2001). Firstly, the fact that in many of the studies, the heterologous expression of UCP 3 in yeast results in a decrease the fully uncoupled respiration rate attained by FCCP (artificial uncoupler). Secondly, the inference in some studies of the presence and activity of the heterologous protein based simply on the phenotype of uncoupling and unknown levels of proteins expressed (Stuart et al., 2001). Rial et al. (1999), has previously shown that retinoic acid increases proton transport in yeast mitochondria expressing UCP 2. Interestingly, Couplan et al. (2002), has shown UCP 2’s lack of sensitivity to retinoic acid, a fatty-acid known to activate UCP 1, in isolated spleen mitochondria. Additionally, Hagen et al. (2000), showed, using heterologous expression systems, that UCP 1 was strongly activated by free fatty-acids whereas no stimulatory effect on UCP 3 was observed.

\[1.10.2\] UCP’s reconstituted in liposomes

An alternative approach taken to study function and regulation of UCP 2 and UCP 3 has been to reconstitute these proteins into liposomes after expression in E. coli (Echtay et al., 1999; Jabů rek et al., 1999). It was shown that, like UCP 1, UCP 2 and UCP 3 catalyze electrophoretic H⁺ flux, which requires the presence of free fatty-acids. Recent data obtained by Jabů rek et al. (1999), reconstituted the transport activity of human UCP 2 and UCP 3 purified from recombinant bacteria into liposomes and observed that the two proteins catalysed an electrophoretic flux of protons, similar to UCP 1. Jabů rek et al. (1999) also reported that fatty-acids were obligatory for the proton transport activity of UCP 2 and UCP 3, again similar to UCP 1. This data led the authors to conclude that UCP 2 and UCP 3 are true uncoupling proteins. Echtay et al. (1999), also reported the reconstitution of the chloride transport activity of UCP 3 and observed an inhibition by purine nucleotides, like UCP 1.

\[1.10.3\] Transgenic overexpression and gene knockout studies

Transgenic mice overexpressing uncoupling proteins and knockout models have been produced as a direct approach to investigate the physiological function of UCP’s. Matthias et al. (1999) and Monemdjou et al. (1999) studied the bioenergetics
of BAT mitochondria from UCP 1-ablated mice. Interestingly, they found that UCP 2 mRNA levels are increased up to 14-fold in the BAT of these mice. BAT mitochondria isolated from UCP 1 ablated mice are innately energized as evidence by a high membrane potential and a low respiration rate. The UCP 1 ablated mice are highly cold-intolerant, and this suggests that the heat production lost by UCP 1 ablation is not compensated by other UCP's. Importantly, neither UCP 2 nor UCP 3 have replaced the proton conductance or thermogenic capacity lost by UCP 1-ablation in these mice (Matthias et al., 1999; Nedergaard et al., 2001 & 2001a).

Transgenic mice that overexpress human UCP 3 in skeletal muscle (UCP 3tg mice) have been created by Clapham et al. (2000). Total UCP 3 expression (mRNA) was increased by 66-fold in skeletal muscle with little or no expression in other tissues. Despite an increase in food intake, UCP 3tg mice weighed less than controls. Skeletal muscle mitochondria isolated from transgenic mice that overexpress human UCP 3 displayed a 2-3-fold increase in proton conductance, compared to control mice (Cadenas et al., 2000). These results are all consistent with a role of UCP 3 in energy expenditure.

More compelling evidence suggesting that UCP 3 is an uncoupling protein came from two recent studies using UCP 3 knockout (KO) mice. Gong et al., (2000), showed that UCP 3 KO are not obese and have a phenotype similar to control mice. Therefore, the lack of UCP 3 is not associated with obesity. Gong et al. (2000) also showed that the proton leak is reduced in skeletal muscle mitochondria isolated from UCP 3 KO mice, thus suggesting that UCP 3 accounts for some of the proton leak in skeletal muscle. Similar results were found by Vidal-Puig et al. (2000), who showed that skeletal muscle mitochondria isolated from UCP 3 KO mice are more coupled, as measured by a respiratory control ratio. These results suggest that the mere presence of UCP 3 leads to uncoupling activity. Both Gong et al. (2000) and Vidal-Puig et al. (2000) showed that mitochondria lacking UCP 3 showed an increased production of reactive oxygen species (ROS) as determined by measurement of superoxide and aconitase activity. It has previously been shown that mitochondrial membrane potential regulates the generation of ROS (Papa et al., 1997). Thus, a partial uncoupling and its associated decrease in membrane potential could lead to a
decreased superoxide production. These results support a role for UCP 3 in preventing excessive oxidative damage in skeletal muscle.

Additionally, it has been shown that UCP 2 acts as an uncoupling protein in macrophages and pancreatic β-cells, thereby postulating a role for UCP 2 as an uncoupler in vivo (Couplon et al., 2002). It has recently been shown that UCP 2 is expressed in pancreatic β-cells (Shimabukuro et al., 1997; Zhou et al., 1997; Chan et al., 1999), raising the possibility that UCP 2 might influence insulin secretion by regulating the amount of ATP derived from metabolized glucose (Boss et al., 1998b). In support of this view, it was observed that adenovirally mediated expression of UCP 2 in pancreatic islets markedly reduced insulin secretion in response to glucose by isolated islets (Chan et al., 1999). Krauss et al., (2002), showed that proton leak activity was lower in thymocytes (expressing UCP 2) from UCP 2-deficient mice compared to wild-type and that fatty-acid analogues (i.e. retinoic acid) activated the proton leak in thymocytes isolated from wild-type mice.

While data from genetically manipulated systems support an uncoupling function for the UCP 1 homologues, experiments on animal models that have been physiologically perturbed do not.

[1.10.4] Evidence against UCP 2 and UCP 3 being uncoupling proteins arising from physiologically perturbed natural models

As previously mentioned (section 1.8), Cadenas et al. (1999) has shown a 2-fold increase in UCP 3 protein expression levels in skeletal muscle mitochondria upon starvation. Despite this increase, the mitochondrial proton conductance in skeletal muscle mitochondria remains unchanged (Cadenas et al., 1999). Similarly, in mice, starvation induced a 3.5-fold increase in soleus UCP 3 mRNA expression but it did not increase soleus muscle heat production in vivo (Boss et al., 1998). Cresenzo et al. (2003), has recently shown that refeeding on a high-fat diet, which exacerbates the suppression of thermogenesis, resulted in a 2-fold elevation in UCP 3 protein but no change in State 4 or State 3 respiration. Taken together, these results show an increase in UCP 3 mRNA and protein expression but show no change in proton conductance, thereby inferring that UCP 3 is not an uncoupling protein. Similarly, Jekabsons et al. (1999) showed that despite altered
UCP 2 and UCP 3 mRNA expression, non-phosphorylating respiration of skeletal muscle mitochondria is unchanged. Again, these results casts doubt on UCP 2 and UCP 3's involvement in physiological uncoupling.

[1.10.5] Evidence against UCP 2 and UCP 3 being uncoupling proteins arising from site-directed mutagenesis studies

A further reason for the lack of uncoupling activity in mitochondria known to contain UCP 2 and UCP 3 has evolved from site-directed mutagenesis studies. In association with the "Proton buffering" model, Bienengraeber et al. (1998), has shown that mutation of two histidine residues in UCP 1 cause loss of proton transport. The authors' interpretation of this result is that His^{145} and His^{147} comprise part of the proton-conductance pathway in UCP 1 (Figure 1.8). As UCP 2 contains neither histidine, these authors conclude that UCP 2 does not conduct protons. Additionally, as UCP 3 contains only one histidine, these authors imply that UCP 3 conducts protons only weakly. This may infer a reason why UCP 2 and UCP 3 cannot conduct protons in vitro. Additionally, these two histidine residues were predicted to be located in the second matrix loop of UCP 1. Interestingly, a study using chimeric proteins by Hagen & Lowell, (2000), has shown that activation of UCP 1 by free fatty-acids is mediated by the second repeated domain, since substitution of the second repeat of UCP 1 by the equivalent repeat of UCP 3 abolishes fatty-acid activation. In contrast, replacing the second repeat domain of UCP 3 by the corresponding repeated domain of UCP 1 results in fatty-acid activation similar to wild-type UCP 1. Taken together, these results indicate that UCP 1 and UCP 3 are regulated differently and furthermore indicate that sequence difference between UCP 1 and UCP 3 may result in the lack of fatty-acid activation observed in skeletal muscle mitochondria known to contain UCP 3.

Correspondingly, Nedergaard et al. (2001a), identified two very specific sequences, 144SHLHGIKP and the C-terminal sequence RQTVDC(A/T)T conserved solely to UCP 1 and not to UCP 2 and UCP 3. These authors further show that the sequence 144SHLHGIKP, which is probably located on the matrix side of the membrane includes the two histidine residues (His^{145} and His^{147}) previously found to be necessary for UCP 1 proton transport but which are not conserved fully in UCP 2.
and UCP 3 (Bienengraeber et al., 1998). Nedergaard et al. (2001a), also postulates that these two sequences may therefore be essential for the unique thermogenic function of UCP 1 and thus the absence of these sequences in UCP 2 and UCP 3 may therefore infer a lack of thermogenic function in UCP 2 and UCP 3.

[1.11] Regulation of proton conductance by UCP 2 and UCP 3

[1.11.1] Purine nucleotide inhibition

As stated previously, UCP 1’s uncoupling activity is regulated negatively by purine nucleotides and positively by free fatty-acids. Our study sought to investigate whether UCP 2 and UCP 3’s physiological function were regulated in a similar manner.

Purine nucleotides are candidate regulators of UCP 2 and UCP 3’s function given that the extensive nucleotide binding domain of UCP 1 is 75-80% conserved in UCP 2 and UCP 3. However, as previously described (section 1.10.1), the marginally higher state 4 rates of yeast mitochondria expressing UCP 2 and UCP 3 are unaffected by 1 mM purine nucleoside di- and tri-phosphates (Hagen et al., 1999; Rial et al., 1999; Zhang et al., 1999; Hagen et al., 2000). In contrast, purine nucleotides inhibit UCP 2 and UCP 3 reconstituted into artificial liposomes either with nM (Echtay et al., 1999) or μM affinity (Jaburek et al., 1999). Despite the presence of UCP 3 in skeletal muscle mitochondria, proton leak is not inhibited by 1 mM purine or pyrimidine nucleoside di- or tri-phosphates (Cadenas et al., 2000; Jekabsons et al., 2001). Similarly, as previously mentioned, BAT isolated from UCP 1 knockout mice exhibits 5-14 fold higher UCP 2 mRNA levels (Enerbäck et al., 1997; Matthias et al., 1999). Despite this, proton leak in BAT mitochondria isolated from UCP 1 knockout mice are insensitive to 1 mM GDP (Monemdjou et al., 1999). Thus, purine nucleotides neither reverse the effects of UCP 2 or UCP 3 expression in yeast mitochondria nor inhibit the basal proton conductance in isolated mitochondria.

[1.11.2] Fatty-acid effects on mitochondria and liposomes containing UCP 2 and UCP 3

Our study sought to investigate whether UCP 2 and UCP 3’s physiological function (unknown) was activated by free fatty-acids.
When reconstituted from BAT or yeast mitochondria, UCP 1 requires exogenous fatty-acids for its activity (Winkler et al., 1994). Although UCP 1 can be activated by fatty-acids of different chain lengths, peak activation occurs with laurate (C\(_{12}\)), myristate (C\(_{14}\)) and palmitate (C\(_{16}\)) (Klingenberger & Huang, 1999). Using bacterially expressed and reconstituted UCP 2 and UCP 3, fatty-acids either have no effect on stimulating proton uncoupling activity (Echtay et al., 1999) or, as with UCP 1 reconstituted from BAT or yeast, are required in order to observe this activity (Jabur et al., 1999). All trans-retinoic acid stimulates respiration of yeast mitochondria expressing UCP 1 and UCP 2 but not UCP 3 (Rial et al., 1999). Oleate is equally effective in uncoupling skeletal muscle mitochondria isolated from fed and fasted rats, despite upregulation of UCP 2 and UCP 3 mRNA and UCP 3 protein expression (Cadenas et al., 1999). Thus there are no data to indicate that the uncoupling effects of fatty-acids or trans-retinoic acid in artificial UCP expression systems is related to the uncoupling effect of fatty-acids in mitochondria of tissues where UCP 2 and UCP 3 are constitutively expressed.

[1.12] Possible functions/roles of UCP’s
[1.12.1] Control of adaptive thermogenesis in response to cold exposure and diet:

UCP 1 gene knockout mice are unable to maintain body temperature during cold exposure, which confirms the long-held view that a major function of UCP 1 is cold-induced nonshivering thermogenesis (Himms-Hagen, 1990; Enerbäck et al., 1997). It has also been suggested that UCP 1, by stimulating facultative diet-induced thermogenesis, plays an important role in regulating body weight (Rothwell & Stock, 1979). As UCP 2 and UCP 3 share amino acid similarity with UCP 1, it is plausible that they also play an important role in regulating adaptive thermogenesis in response to cold and diet. However, powerful evidence against this view is the observation that UCP 2 mRNA levels and UCP 3 mRNA and protein levels are markedly increased during starvation (Boss et al., 1998; Weigle et al., 1998; Brun et al., 1999; Cadenas et al., 1999; Sivitz et al., 1999; Samec et al., 1999; Moreno et al., 2003), a time when whole-body energy expenditure is reduced (Rothwell et al., 1984). Excitingly, it has been shown that ecstasy induced a UCP 3 dependent
thermogenicity in mouse skeletal muscle (Mills et al., 2003). However, despite some evidence to suggest that UCP 2 and UCP 3, akin to UCP 1 are uncoupling proteins, there is as yet little evidence to suggest that UCP 2 and UCP 3 can regulate adaptive thermogenesis. Given this, it is important to consider other possible functions for UCP 2 and UCP 3.

[1.12.2] Control of reactive oxygen species production

Excluding phagocytes, the majority of reactive oxygen species (ROS) are generated by the mitochondrial electron transport chain (Chance et al., 1979), possibly by reaction of oxygen with the semiquinone radical (Skulachev, 1996). Using isolated mitochondria, it has been shown that production of ROS is greatly increased at times when the proton electrochemical gradient is high. Such conditions occur during State 4 respiration when ADP is unavailable. Addition of either ADP, which induces proton transport via the ATP synthase, or an uncoupling agent, which directly decreases the proton electrochemical gradient strongly suppresses superoxide anion formation (Skulachev, 1996). In fact, uncouplers increase the rate of electron transfer and inhibit superoxide formation by mitochondria (Chance et al., 1979). The explanation of this effect is that partial uncoupling stimulates oxygen consumption, shortens the lifetime of CoQ⁻ and inhibits ROS production (Skulachev, 1996). Therefore, uncoupling represents a powerful system for the limitation of ROS production. This suggested that one of the functions of proteins that uncouple respiration (i.e. UCP’s) could be the limitation of ROS synthesis. Interestingly, Nègre-Salvayre et al. (1997) showed that H₂O₂ production was stimulated by the inhibition of BAT, spleen and thymus mitochondrial respiration through the addition of GDP, which directly inhibits UCP’s, thereby indicating that UCP’s may be regulators of H₂O₂ production in the BAT, spleen and thymus. In other studies, UCP 2 expressed in spleen have been associated with a limitation of ROS production (Arsenijevic et al., 2000; Vidal-Puig, 2000), thereby supporting the idea that UCP 2 functions to limit ROS production. As stated previously, Gong et al. (2000) and Vidal-Puig et al. (2000) showed that mitochondria lacking UCP 3 showed an increased production of reactive oxygen species (ROS). These results also support a role for UCP 3 in preventing excessive oxidative damage in skeletal muscle.
mitochondria. More recently, it has been shown that there is an inducible (GDP-sensitive) uncoupling of BAT, spleen and skeletal muscle mitochondria associated with UCP 1, UCP 2 and UCP 3, respectively, under conditions of continuous intramitochondrial and extramitochondrial superoxide production (Echtay et al., 2002 & 2002a; Echtay et al., 2003; Murphy et al., 2003). Taken together, these results postulate a role for the UCP’s in regulating ROS production by mitochondria.

[1.12.3] Regulation of free fatty-acid oxidation/metabolism

Induction of UCP 2, and especially UCP 3 mRNA levels in states when fatty-acid β-oxidation is likely to be increased raises the possibility that UCP 2 and/or UCP 3 are required for β-oxidation or that UCP 2 and/or UCP 3 protect cells from consequences of excessive fat metabolism or storage (Wang et al., 1999). The association of UCP 2 with fat metabolism comes from observations of increased UCP 2 mRNA levels in WAT of some obese mouse models (Gimeno et al., 1997), brown adipocytes of UCP 1 gene knockout mice that have increased lipid storage (Enerbäck et al., 1997) and hepatocytes of ob/ob mice that develop hepatic steatosis (Chavin et al., 1999). In skeletal muscle, physiological states associated with enhanced fat metabolism are correlated with increased UCP 3 mRNA and protein levels. Such situations include starvation, cold-exposure, thyroid treatment and intralipid plus heparin injections to rats, which increase circulating free fatty-acid levels (Weigle et al., 1998; Simonyan et al., 2001; Lombardi et al., 2002). Perhaps the most compelling association comes from newborn mice, which do not express UCP 3 in skeletal muscle until suckling occurs (i.e. ingestion of a fat-rich meal) (Brun et al., 1999).

Dulloo et al. (2001), has proposed that the primary function of UCP 3 in muscle and BAT maybe involved with the regulation of lipids as fuel substrate and/or in controlling the export of fatty-acids out of the mitochondria during fatty-acid oxidation, rather than the mediation of thermogenesis. Several lines of evidence support his proposal. Firstly, the observations that changes in UCP 3 gene expression during starvation and refeeding are more pronounced in white muscles (i.e fast glycolytic) than in red muscles (i.e slow oxidative) are consistent with the greater
dependency of slow-oxidative muscles on lipids as fuel substrate, and the greater shift between glucose and lipids as fuel substrate in fast glycolytic muscles during fasting and refeeding (Samec et al., 1998). Schrauwen et al. (2001), showed that high-fat induced up-regulation of skeletal muscle UCP 3 gene expression is more pronounced in humans with a high proportion of glycolytic fibres than in slow oxidative muscle fibres. Therefore, muscle-type differences in dietary regulation of UCP 3 expression, are coherent with UCP 3’s role in lipid utilization.

Vidal-Puig et al. (2000) showed that mice lacking UCP 3 had impaired starvation-induced shift in lipid partitioning between oxidation and storage, reflecting perhaps the tendency for compensatory mechanisms to counter, almost completely the consequences of a lack of UCP 3. Harper et al. (2001) hypothesized that UCP 3 could facilitate rapid rates of fatty-acid oxidation by acting as a mitochondrial fatty-acid efflux protein. Harper et al. (2001) proposed that UCP 3 functions in concert with mitochondrial thioesterase(s) (MTE) to remove free fatty-acids (produced by MTE) from the matrix and liberate CoA. The latter is in relatively high demand during fatty-acid oxidation. It is also interesting to note that agonists of peroxisome proliferator-activated receptor (PPAR)-γ and PPAR-α, two transcription factors regulated by lipid metabolites have been shown to regulate the expression of UCP 2 and UCP 3 (Nagase et al., 1999; Cabrero et al., 2000a; Teruel et al., 2000). Taken together, these results all postulate possible roles for UCP 2 and UCP 3 in fatty-acid oxidation/metabolism.

[1.13] UCP’s and the immune system

As already mentioned, UCP 2 is the only UCP associated with cells and tissues of the immune systems. As previously stated, UCP 2 mRNA is expressed in human and rat thymus and spleen (Nègre-Salvayre et al., 1997; Gong et al., 1997; Richard et al., 1999; Ricquier & Bouillaud, 2000; Jekabsons et al., 2001; Pecqueur et al., 2001; Horvath et al., 2003).

The thymus is a primary lymphoid organ which plays a central role in the creation of a fully functional immune system. Its major function is to provide the appropriate milieu within which cells of the T-lymphocyte lineage can develop,
proliferate, mature and generate their antigen receptor repertoire (Ritter & Crispe, 1992). On the other hand, the spleen is the largest lymphatic organ, with its cellular components mainly consisting of lymphocytes, macrophages and monocytes (MacPherson, 1973). The spleen is also known to contain a small number of mitochondrial-containing reticulocytes (Takano-Ohmuro et al., 2000). One of the main functions of the spleen is to bring blood into contact with lymphocytes. As blood flows slowly through the spleen, any disease organisms within it are likely to come into contact with lymphocytes in the spleen tissue. This contact activates the lymphocytes, which can then attack the foreign invaders. Finally, it is known that lymphoid atrophy is a well-recognised consequence of nutritional deprivation in animals and human which results in the shrinkage of both the spleen and thymus (Howard et al., 1999).

The exact function or role of UCP 2 in these immune tissues has yet to be elucidated. One may postulate that UCP 2 may be involved in T-lymphocyte development, proliferation and metabolism. Similarly, apoptosis is a prolific event in the lifetime of the thymus (Ritter and Crispe, 1992) so one may postulate that UCP 2 is involved in apoptosis in the thymus. Krauss et al. (2002) has shown that UCP 2, expressed at endogenous levels, mediates proton leak in intact thymocytes, thereby implying a role for UCP 2 as an uncoupling protein in the thymus. Nègre-Salvayre et al. (1997) has shown UCP 2 may regulate H$_2$O$_2$ production in the thymus and spleen.
[1.14] Aims of this study

➤ To investigate the mRNA expression patterns of UCP’s in a variety of tissues using reverse transcriptase polymerase chain reaction (RT-PCR)

As there are very few publications showing protein expression and functional data of the UCP proteins in natural systems, we sought to investigate:

➤ UCP protein expression and factors affecting its expression in isolated mitochondria from a variety of tissues

➤ Whether UCP’s can bind purine nucleotides in isolated mitochondria known to contain UCP 1, UCP 2 and UCP 3

➤ Whether UCP’s are uncoupling proteins in isolated mitochondria and if their uncoupling activity is regulated positively by fatty-acids and negatively by purine nucleotides.
Chapter 2

Materials & Methods
Chapter 2
Materials and Methods

[2.1] Materials

Aerosol barrier tips
AMV-RT
AM580
BioBeads
BSA (de-fatted)
Commercial peptide antibodies
Coomassie Blue R
DEPC
DNA Ladder (1 Kb)
Dowex
ECL Western Blotting Reagents
Ecoscint\textsuperscript{TM}
Fetal Calf Serum
Folins-Ciocalteau Phenol Reagent
$[^3]$H GDP
GDP
Grabit Annotating Grabber software
HISTOPAQUE\textsuperscript{®}-1077
Hydroxyapatite
Human Spleen cDNA library
Human Thymus cDNA library
Peptide generated antibodies
\text{L-Glutamine}
MJ Research Minicycler thermocycler
Mini Protean\textsuperscript{®} 3 Cell
Nagarse
Octyl-POE

M\beta P Molecular BioProducts
Promega
Tocris
Bio-Rad
Sigma
Calbiochem
Sigma
Sigma
Promega
Fluka
Amersham International
National Diagnostics
Sigma
Merck
Amersham International
Sigma
UVP Ltd
Sigma
Bio-Rad
Clontech
Genemed
Eurogentec
Sigma
Biosciences
Bio-Rad
Sigma
Bachem
Palmitate Sigma
PCR Primers MWG Biotech
Protein A Sigma
Protein markers New England Biolabs
PVDF Millipore
Retinoic Acid Tocris
RNase-free plasticware Sarstedt
RPMI-1640 Sigma
Scion Imaging Software Scion
SERVA Blue G SERVA
Sonicator (Branson 200) Lennox
[¹⁴C] Sucrose Amersham International
Taq DNA Polymerase Promega
TRI-REAGENT Sigma
UV Transilluminator UVP Ltd
Whatman Glass fibre filter paper (GF/C) Whatman
X-Omat™ LS film Kodak

All other reagents and equipment were of molecular biology or analytical grade and were obtained from Sigma, BDH or Merck.

Addresses of Suppliers:
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Biosciences, 3 Charlemont tce, Crofton Road, Dunlaoghaire, Co. Dublin, Ireland.
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Eurogentec, Parc Scientifique du Sart Tilman, 4102 Seraing, Belgium

Kodak, Eastman Kodak Company, Rochester, New York 14650, U.S.A.

Fluka, c/o Sigma-Aldrich Ireland Ltd., Dublin, Ireland.

Genemed Biotechnologies, Inc., 458 Carlton Court, South San Francisco, CA 94080, USA.

Lennox, JFK Drive, Naas Road, Dublin 12, Ireland

Merek, Merck KgaA, D-64271 Darmstadt, Germany.

Millipore, The Boulevard, Blackmoor Lane, Watford, Hertfordshire WD1 8YW, U.K.

Molecular BioProducts (MPB), 9880 Mesa Rim Road, San Diego, California 92121, U.S.A.

M.W.G. Biotech UK Ltd., Waterside House, Peartree Bridge, Milton Keynes, MK6 3BY, U.K.

National Diagnostics, 305 Patton Drive, Atlanta, Georgia 30336, U.S.A


Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711-5399, U.S.A

Sarstedt Ltd., 68 Boston Road, Leicester, Leicestershire, LE4 1AW, U.K.

Scion Corporation, Maryland, U.S.A.

SERVA Electrophoresis, GmbH, Carl-Benzi-Str. 7, P.O.B 105260, 69115 Heidelberg, Germany.

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, U.K.

Tocris Cookstown Ltd., Langford, Bristol, B540 SDW, U.K.


Whatmann International Ltd., Whatman House, St Leonard’s Road, 20/20 Maidstone, Kent, SG1 OLS, U.K.
Chapter 2
Methods

[2.2]: Precautions taken when attempting to work in an RNase-free environment

RNases (ribonucleases) are particularly hardy enzymes and are difficult to inhibit. Degradation of RNA by ribonucleases (RNases) is minimized by a number of precautions taken prior to RNA extraction. Such precautions are as follows:

Glassware and plasticware: Laboratory glassware and plasticware can be a source of RNase contamination. All glassware, metal spatulas and ultra-turrax probes were baked at 180°C for 12 hours in order to completely inactivate any RNases. Sterile disposable plasticware is generally considered to be RNase-free but as a precaution, was autoclaved for 30 minutes at 121°C before use. Similarly, pre-sterile, aerosol barrier RNase free pipette tips were used at all times. Microcentrifuge tubes (Eppendorf) were removed from sealed packaging (using disposable gloves), placed into heat-treated glassware and autoclaved for 30 minutes at 121°C prior to use. All manipulations were performed at room temperature unless otherwise stated.

Use of disposable gloves: Disposable gloves were used at all times when handling glassware, plasticware or preparing solutions and samples. Gloves are RNase free but are easily contaminated with non-sterile lab equipment, reagents and clothing. Consequently, gloves were changed very frequently, as hands are a major source of RNase contamination.

Preparation of solutions: All solutions were prepared with RNase-free chemicals and water. All chemicals were purchased for the sole use of RNA work and thus were kept free of contaminating RNases. Chemicals were weighed out using RNase-free spatula’s in a fume-hood. Disposable gloves were used at all times when handling chemicals for making solutions. Water was treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) for 12 hours in fume hood and autoclaved for 30 minutes at 121°C to remove any traces of DEPC. This DEPC-H$_2$O was used to make any solutions required for RNA isolation, RT-PCR and PCR analysis.
Total RNA was purified using a TRI REAGENT™ (Sigma). This system employs guanidine thiocyanate to inactivate RNases and disrupt nucleoprotein complexes. A phenol-chloroform extraction partitions the RNA in the aqueous phase and is concentrated by precipitation with isopropanol.

Sample preparation: A section of brown adipose tissue (BAT), thymus and liver was extracted rapidly from a rat and placed into a sterile minifuge tube containing the TRI REAGENT™. The sample was left on ice prior to homogenisation. The tissue was then homogenized in TRI REAGENT™ (1 ml per 50-100 mg of tissue) using an RNase-free ultra-turrax probe. To permit the complete dissociation of nucleoprotein complexes, the homogenised samples were left for 5 minutes at room temperature. 0.2 ml of chloroform was added to the sample per ml of TRI REAGENT™ used. The sample was shaken vigorously for 15 seconds and allowed to stand for 15 minutes at room temperature. The resulting mixture was centrifuged at 12,000 x g for 15 minutes at 4°C. This centrifugation step separates the mixture into three phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA).

RNA Precipitation: The upper aqueous phase (containing RNA) was transferred to a fresh sterile minifuge tube with the addition of 0.5 ml isopropanol per ml of TRI REAGENT™. The sample was mixed and allowed to stand at room temperature for 10 minutes at 4°C. The sample was then centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA precipitate forms as a pellet on the side and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol (per ml of TRI REAGENT™ used in sample preparation). The sample was vortexed and then centrifuged at 7,500 x g for 5 minutes at 4°C. The RNA pellet was briefly air-dried in a fume-hood for 10 minutes at room temperature. The pellet was resuspended in 0.05 ml of DEPC-H₂O and incubated at 55-60°C for 10 minutes, before storing at -20°C.
[2.4] Quantification and Analysis of Total RNA

**Quantification:** The yield of RNA was determined spectrophotometrically at 260 nm using the formula:

\[
1 \text{ A}_{260} \text{ nm unit} = 40 \mu g \text{ RNA/ml.}
\]

Samples were diluted 1 in 400 with DEPC-H$_2$O to a final volume of 1 ml and measured at both \( \text{A}_{260} \text{ nm} \) and \( \text{A}_{280} \text{ nm} \). Pure total RNA will exhibit an \( \text{A}_{260}/\text{A}_{280} \) ratio of between 1.7-2.0. A ratio lower than this is generally indicative of contamination with guanidine thiocyanate, which may be carried over during the precipitation steps. Only total RNA with an \( \text{A}_{260}/\text{A}_{280} \) ratio >1.7 was used for RT-PCR analysis.

**Integrity:** The integrity of the RNA was analysed by agarose gel electrophoresis in the presence of formaldehyde. Formaldehyde gel running buffer was made up (10X concentration), so that the final solution contained 0.2 M 3-(N-morpholino) propanesulfonic acid (MOPS); 50 mM sodium acetate, pH 7; 10 mM EDTA. The 1% agarose gel was prepared by melting 0.5 g agarose in DEPC-H$_2$O (50 ml), cooling it to 60°C and adding 10 X formaldehyde gel-running buffer, and formaldehyde, to a final concentration of 1 X and 2.2 M, respectively. This solution was mixed for 30 seconds and then poured into the gel mould of an electrophoresis apparatus (Hybaid) and allowed to set at room temperature in a fume hood for up to 1 hour. Once set, the gel was pre-run for 20 minutes at 60 V in 1 X formaldehyde gel-running buffer. 5 \( \mu g \) of totRNA was dissolved in 1 X sample buffer (0.75 ml formamide, 0.15 ml 10 X Mops, 0.24 ml formaldehyde, 0.1 ml DEPC-H$_2$O, 0.1 ml glycerol and 0.08 ml of 10% (w/v) bromophenol blue). All samples were mixed gently, pulsed in minifuge and heated to 60°C for 10 minutes. 2 \( \mu l \) of a 1 \( \mu g/ml \) stock of ethidium bromide (EtBr) was added to all samples and vortexed briefly. Samples were then immediately loaded on to agarose gel and ran for 4 hours at 60 V. The RNA was visualized using a u.v transilluminator. A 2:1 ratio of the 28S:18S ribosomal RNA band intensities indicate that the total RNA is intact and that no significant RNA degradation has occurred.
Reverse Transcription of Total RNA

Reverse Transcription Polymerase Chain Reaction (RT-PCR) was the method used in this study to determine expression levels of UCP 1, UCP 2 and UCP 3 mRNA, relative to the ubiquitously-expressed housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A flow chart illustrating the RT-PCR process is shown in Figure 2.1. Random hexamer primers were used to synthesise cDNA from total RNA, which was then used as a PCR template.

First strand complimentary DNA (cDNA) was synthesized from mRNA contained in the total RNA preparation using avian myeloblastosis virus reverse transcriptase (AMV-RT). The reaction primers were a random hexamer oligonucleotide mixture. The reactions were prepared in 0.5 ml eppendorf tubes and the reactions were incubated in an MJ Research Minicycler™ thermocycler. The reaction details are set out as follows and 1µg of totRNA was used as a template. All reagents were supplied from Promega, unless otherwise stated.

The following components were prepared in a 0.5 ml eppendorf tube:

<table>
<thead>
<tr>
<th></th>
<th>Volume (µl)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂, 25mM</td>
<td>4.0</td>
<td>5mM</td>
</tr>
<tr>
<td>5 X reaction buffer (50mM Tris-HCl pH 9, 250mM KCl, 0.5% Triton® X-100)</td>
<td>4.0</td>
<td>1 X</td>
</tr>
<tr>
<td>Deoxyribonucleoside triphosphates (dNTP's) (10mM each of dATP, dTTP, dCTP, dGTP) (Sigma)</td>
<td>2.0</td>
<td>1mM each</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>0.5</td>
<td>1 U/µl</td>
</tr>
<tr>
<td>AMV-RT</td>
<td>1.0</td>
<td>0.75U/µl</td>
</tr>
<tr>
<td>Random primers</td>
<td>1.0</td>
<td>0.5µg/ml</td>
</tr>
<tr>
<td>Template (totRNA)</td>
<td>1.0µg</td>
<td></td>
</tr>
<tr>
<td>DEPC-H₂O</td>
<td></td>
<td>up to a total volume of 20µl</td>
</tr>
</tbody>
</table>
RNA is first isolated from cells or tissues of interest and is then used as a template for reverse transcription to complimentary DNA (cDNA). The cDNA is used as the template for PCR, using primers designed to amplify a selected cDNA region. Following PCR, the product is typically analyzed by agarose gel electrophoresis. The amplified PCR product is identified by the size of the PCR product, which is predicted from the knowledge of the cDNA nucleotide sequence.
1 μg of total RNA was incubated at 70°C for 10 minutes to remove any secondary structure, and cooled on ice prior to addition in reaction mixture. Two RT-PCR control reactions, encompassing DEPC-H$_2$O as a template in lieu of total RNA and DEPC-H$_2$O in place of AMV-RT, were carried out to screen for contamination in parallel. Samples were incubated in the thermocycler at 18°C for 10 minutes, 42°C for 60 minutes, 99°C for 5 minutes (to inactivate the AMV-RT enzyme) and cooled at 4°C before PCR analysis or storing at -20°C.

[2.6] Polymerase Chain Reaction (PCR)

A Polymerase Chain Reaction (PCR) was used to amplify cDNA using Taq DNA polymerase. Oligonucleotide primers specific for the gene of interest (Table 2.1) were used to initiate amplification. The reactions were typically prepared in 0.5 ml eppendorf tubes and incubated in a MJ Research Minicycler™ thermocycler. All reagents were supplied by Promega unless otherwise stated.

The following components were prepared in a 0.5 ml eppendorf tube:

<table>
<thead>
<tr>
<th></th>
<th>Volume (μl)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl$_2$, 25mM</td>
<td>5.0</td>
<td>2.5mM</td>
</tr>
<tr>
<td>10 X Reaction buffer (100mM Tris-HCl pH 9, 500mM KCl, 1% Triton® X 100)</td>
<td>5.0</td>
<td>1.0 X</td>
</tr>
<tr>
<td>Deoxyribonucleoside triphosphates (dNTP's) (10mM each of dATP, dTTP, dCTP, dGTP) (Sigma)</td>
<td>1.0</td>
<td>0.2mM each</td>
</tr>
<tr>
<td>Forward primer (see Table 2.1)</td>
<td>5.0</td>
<td>1.0μM</td>
</tr>
<tr>
<td>Reverse primer (see Table 2.1)</td>
<td>5.0</td>
<td>1.0μM</td>
</tr>
<tr>
<td>Template (cDNA)</td>
<td>3.0-5.0</td>
<td></td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5</td>
<td>2.5U</td>
</tr>
<tr>
<td>DEPC-H$_2$O</td>
<td>Up to final</td>
<td>volume of 50μl</td>
</tr>
<tr>
<td>Gene</td>
<td>Primers (5'-3')</td>
<td>Annealing Temp</td>
</tr>
<tr>
<td>------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>UCP1</td>
<td>CTCACCTTTGAGCTCCTCCTGGATG</td>
<td>54.9°C</td>
</tr>
<tr>
<td>UCP2</td>
<td>CGAAGCCTACAAGACCATTGAGCATGGTCAGGGCACAGTG</td>
<td>59.4°C</td>
</tr>
<tr>
<td>UCP3</td>
<td>GAGAACCCAGGAGTGCAGAGCTGGCATGGTTCTGTAGGC</td>
<td>61.4°C</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACCCCTTCATTGACCTCAACTACAAGTGATGGCATGGACTGTGGTCAT</td>
<td>61.9°C</td>
</tr>
</tbody>
</table>

Table 2.1: Primer sequences used for PCR analysis.

The sequence of the forward and reverse oligonucleotide primers used for RT-PCR and PCR analysis of gene expression. Optimum annealing temperatures, amplification product size and GenBank accession numbers are shown. Primers were supplied by MWG Biotech.
The Taq DNA polymerase was the last component added to the reaction mixture. On addition of Taq, samples were pulsed briefly and incubated at 4°C prior to amplification. Two control PCR reactions, encompassing DEPC-H2O as a template in lieu of cDNA and DEPC-H2O in place of Taq DNA polymerase, were carried out in parallel to screen for PCR contamination. The reaction mixtures were overlaid with 0.03 mls of mineral oil (Sigma), only in the absence of a heated lid on the thermocycler. Otherwise, samples were amplified using a MJ Research Minicycler™ thermocycler under the following conditions exemplified in Figure 2.2.

![PCR programme](image)

**Figure 2.2:** A typical PCR programme for the amplification of UCP 1, UCP 2, UCP 3 and GAPDH cDNA.

The PCR products were analysed using agarose gel electrophoresis. The PCR products were analysed on a 1% agarose gel containing ethidium bromide (EtBr) (2µl/50ml gel). 10 µls of each PCR product obtained, were dissolved in 2µls of sample buffer (6 X: 10 mM Tris-HCl, pH 7.5, 50 mM EDTA, 10% Ficoll® X 400, 0.2% xylene cyanol SS) and immediately loaded onto the agarose gel. The agarose gel was ran at 100 V for 45 minutes in TAE buffer (40 mM Tris-HCl, pH 7.2, 1.2 mM Glacial acetic acid, 1.0 mM EDTA). PCR products were analysed using a u.v transilluminator.
[2.7]: Animals

[2.7.1] Source: Wistar Rats and mice were bred and obtained from the Bioresources Unit, Department of Biochemistry, Trinity College, Dublin. All animals were kept at room temperature, unless otherwise stated.

[2.7.2] Treatments:

[2.7.21] 48-hour starvation: Wistar rats (200-250 grams) were deprived of food for 48-hours and allowed water ad libitum.

[2.7.22] Temperature acclimation: Wistar rats (200-250 grams) were housed singly in cages and placed in either a cold room set between 6°C ± 2°C or warm room set a 30°C ± 2°C for 28 days. All animals were allowed free access to rat laboratory chow and water.

[2.7.23] Thyroid Treatment: Hyperthyroidism was induced in Wistar rats (200-250 grams) by seven daily i.p. injections of 15 μg T3/100 grams of body weight. Euthyroid control rats were housed similarly and received daily injections of saline.

All animals were killed by cervical dislocation.

[2.8] Mitochondrial isolations:

[2.8.1]: Isolation of Brown Adipose Tissue Mitochondria

BAT was prepared by the method of Scarpace et al. (1991). BAT was removed from the interscapular region of the rat between the head and shoulder blades. BAT was then placed into a pre-weighed 50 ml beaker containing 30 mls of ice-cold (0-4°C) STE buffer (250 mM Sucrose, 5 mM Trizma-base, 1 mM EGTA, pH 7.4). BAT was weighed and washed several times with STE buffer. BAT was chopped carefully in a beaker and poured into a Potter homogeniser tube to a final volume of about 20 mls. The tissue was then homogenised by hand with four passes using a pestle of 0.26 inch (loose) clearance, followed by homogenisation by hand with 6 passes using a pestle of 0.12 inch (tight) clearance. The homogenate was then filtered through 4 layers of muslin and the filtrate was centrifuged at 8,600 x g for 10
minutes at 4°C. The supernatant was discarded and the sides of the centrifuge tubes were wiped with tissue to remove any fat deposits. The pellet was resuspended using a cold-finger (a loose-fitting test-tube filled with ice), that had been suspended in STE buffer, and centrifuged at 750 x g for 10 minutes at 4°C. The pellet was discarded and the supernatant was centrifuged at 8,600 x g for 10 minutes. The pellet was resuspended in STE buffer with the addition of 2% (w/v) de-fatted BSA using a cold finger that had previously been suspended in STE buffer (containing fatty-acid free BSA) and centrifuged at 8,600 x g for 10 minutes. The pellet was then resuspended in STE buffer and centrifuged as above. The resulting mitochondrial pellet was resuspended gently and thoroughly using a cold-finger with 0.05 ml of STE buffer per weight (gram) of original tissue and used within 6-8 hours of isolation.

[2.8.2] Isolation of Skeletal Muscle and Heart Mitochondria

Skeletal muscle mitochondria were isolated from rat hind leg muscle and rat heart mitochondria were isolated by the method of Bhattacharya et al. (1991). 20-30 grams of hindquarter muscle (all leg and a small amount of back muscle) and hearts were removed and placed into a pre-weighed beaker containing 75 mls of ice-cold (0-4°C) isolation medium (100 mM Sucrose, 9 mM EDTA, 100 mM Trizma-base, 46 mM KCl, pH 7.4). Skeletal muscle and heart were weighed and rinsed a few times with ice-cold medium. Muscle and heart were finely chopped on a pre-cooled glass tile with a razor blade, then added to 200 mls of isolation medium containing 0.5% (w/v) BSA and 0.02 % (w/v) nagarse and incubated on ice, with stirring for 5 minutes. The tissues were further disrupted using a Waring commercial blender at half-max speed for 15 seconds. The homogenates were then centrifuged at 1,500 x g for 3 minutes at 4°C. The layer of BSA on top of the supernatants were removed using a spatula and the supernatants were centrifuged at 12,000 x g for 10 minutes at 4°C. The pellets were then resuspended in ~25mls of ice-cold isolation medium (± 2% (w/v) de-fatted BSA) and were re-centrifuged at 12,000 x g for 10 minutes and 4°C. The mitochondrial pellets were resuspended in ice-cold isolation medium and centrifuged as above. The final mitochondrial pellets were resuspended in ice-cold
isolation medium to the desired concentration and stored on ice. Skeletal muscle and heart mitochondria were used within 6-8 hours of isolation.

[2.8.3] Isolation of Liver, Brain, Kidney and Spleen Mitochondria

Mitochondria were prepared essentially by the method of Chappell and Hansford, (1972). Tissues were removed, trimmed of connective tissue and fat and placed into a pre-weighed beaker containing 50 mls of ice-cold (0-4°C) STE buffer (250 mM Sucrose, 5 mM Trizma-base, 2 mM EGTA, pH 7.4). Tissues were weighed, chopped finely using a scissors and washed several times with ice-cold STE buffer. The tissues were poured into a Potter homogeniser tube to a final volume of about 40 mls. The tissues were then homogenised by hand, with 4 passes using a pestle of 0.26 inch (loose) clearance followed by homogenisation by hand with 6 passes using a pestle of 0.12 inch (tight) clearance. The homogenates were centrifuged at 800 x g for 3 minutes at 4°C, pelleting blood and debris. The pellets were discarded and the supernatants were centrifuged at 12,000 x g for 10 minutes at 4°C yielding a “mitochondrial” pellet. The supernatants were discarded and the pellets were resuspended in ~25 mls of STE buffer (± 2% (w/v) de-fatted BSA) and re-centrifuged at 12,000 x g for 10 minutes at 4°C. The pellets were resuspended in STE buffer and centrifuged as above. The resulting pellets containing the mitochondrial fraction were resuspended in STE buffer to the desired concentration and stored on ice. Liver, Kidney, Lung and Brain mitochondria were used within 6-8 hour of isolation.

[2.8.4] Isolation of thymus mitochondria

Thymus mitochondria were prepared essentially by the method of Chappell and Hansford (1972). Any BAT present in the vicinity of the thymus was clearly visible and distinguishable from the thymus and was removed prior to removal of the thymus. The thymus was removed from the abdominal cavity, trimmed of connective tissue and fat and placed into a beaker containing ice-cold (0-4°C) STE buffer (250 mM Sucrose, 5 mM Trizma-base, 2 mM EGTA, pH 7.4). The thymus was weighed, chopped finely using a scissors and washed several times with ice-cold STE buffer. The tissue was poured into a Potter homogeniser tube to a final volume of about 40
The tissue was then homogenised by hand, with 4 passes using a pestle of 0.26 inch (loose) clearance followed by homogenisation by hand with 6 passes using a pestle of 0.12 inch (tight) clearance. The homogenate was centrifuged at 800 x g for 3 minutes at 4°C, pelleting blood and debris. The pellet was discarded and the supernatant was centrifuged at 12,000 x g for 10 minutes at 4°C yielding a "mitochondrial" pellet. The supernatant was discarded and the pellet was resuspended in STE buffer and re-centrifuged at 12,000 x g for 10 minutes at 4°C. The pellet was resuspended in buffer and centrifuged as above. The resulting pellet containing the mitochondrial fraction was resuspended in STE buffer to the desired concentration and stored on ice. Thymus mitochondria were used within 6-8 hour of isolation.

[2.9] Isolation of Thymus cells (Thymocytes)

Thymocytes were isolated from the thymus as described by Buttgereit et al. (1994). An illustration depicting the method used to isolate thymus cells is shown in Figure 2.3. The thymus was removed from the rat, trimmed clean of connective tissue and brown fat and transferred to RMPI-1640 medium containing 10% foetal calf serum (FCS) and L-glutamine. The thymus and medium were poured onto a nylon mesh (tea-strainer will suffice) in a petri dish and, using a plunger of a 5 ml syringe, the thymus was disaggregated into a suspension of thymus cells. This thymus suspension was transferred to a 15ml centrifuge tube, using a Pasteur pipet, and allowed to stand for 10 minutes at room temperature, thus ensuring that debris and cell clumps settle to the bottom of the centrifuge tube. The thymus cells (thymocytes) in the supernatant were aspirated off and transferred to a fresh 15 ml centrifuge tube. The thymus cells were centrifuged at 300 x g for 5 minutes in a bench-top centrifuge. Any fat droplets present on the surface of the supernatant were removed. Similarly, the supernatant was discarded and the thymocyte pellet was resuspended in RMPI-1640 (10% FCS, L-Glutamine), labeled and aliquoted for western blot analysis. Prior to western blot analysis, thymocytes were centrifuged at 300 x g for 5 minutes, and washed twice in phosphate buffered saline (PBS) prior to mitochondrial isolation.
Thymus and medium were poured onto a wire mesh. Thymus was removed from the rat and placed in RPMI-1640 (10% FCS, L-Glut). The thymus cell suspension was transferred to a fresh tube and allowed to settle for 10 min at RT. Using the plunger of a 5 ml syringe, the thymus was disaggregated into a suspension of thymus cells. Connective tissue and epithelial cells (discarded). Supernatant transferred to a fresh tube. Any fat cells were removed. Spun at 300 x g for 5 mins. Supernatant was discarded. Thymus cells (thymocytes). Thymus cells were washed twice in PBS. Supernatant was discarded and the pellet was resuspended in either lysis buffer so as to prepare cell lysates or STE buffer for mitochondrial isolation.

Figure 2.3: Schematic illustration of the method used to isolate thymus cells (thymocytes).
Isolation of Spleen cells

Spleen cells were isolated according to the method of Mills et al. (1996). An illustration depicting the method used to isolate spleen cells is shown in Figure 2.4. Spleen(s) were removed from the abdominal cavity, trimmed free of connective tissue and transferred to a RMPI-1640 medium containing 10% foetal calf serum (FCS) and L-glutamine. The spleen(s) and medium were poured onto a nylon mesh (tea-strainer will suffice) in a petri dish and, using a plunger of a 5 ml syringe, the spleen(s) were grounded until a fine suspension of spleen cells were obtained. This spleen suspension was transferred to a 15 ml centrifuge tube and allowed to stand for 10 minutes at room temperature, thus ensuring that debris and cell clumps settle to the bottom of the centrifuge tube. The spleen cells in the supernatant were aspirated off and transferred to a fresh 15 ml centrifuge tube. The spleen cells were centrifuged at 300 x g for 5 minutes in a bench-top centrifuge. The pellet was re-suspended in RPMI-1640 containing 10% FCS and L-glutamine. 2 ml of this resuspended pellet was labeled (spleen cell homogenate) and aliquoted for western blot analysis.

Cell count: Cells were counted using the Trypan Blue exclusion method. 0.05 ml of 0.1% trypan blue, 0.03 ml RPMI-1640 (10% FCS and L-glutamine) and 0.02 ml of cell suspension were added together and left at room temperature for 10 minutes prior to counting. Cell counting was performed using a haemocytometer.

Purification of the Mononuclear cells from Spleen

Mononuclear cells were isolated from total spleen cells using HISTOPAQUE®-1077. Disposable non-powdered gloves were used at all times as glove powder can activate monocytes and cause lower cell yields. To a 15 ml centrifuge tube, 4 ml of HISTOPAQUE®-1077 was added and allowed to come to room temperature. 8 ml of spleen cell suspension was then carefully layered onto the HISTOPAQUE®-1077, ensuring not to mix the two layers. The mixture was centrifuged at 400 x g for 30 minutes (with no brake) at room temperature. After centrifugation, an upper layer (plasma), an opaque interface (lymphocytes and monocytes) and a pellet (red cells) was observed. The upper layer (containing plasma) to within 0.5 cm of the opaque interface containing mononuclear cells, was
Spleen(s) and medium were poured onto a wire mesh. Spleen(s) were removed from the rat and placed in RPMI-1640 (10% FCS, L-Glut). Using the plunger of a 5 ml syringe, the spleen was grounded to a fine suspension. Cell suspension was transferred to a fresh tube and allowed to settle for 10 min at RT. Cell debris (discarded). Cell debris was discarded. Pellet was resuspended in ~10mls of RPMI-1640.

Homogenate was layered onto Histopaque (1:2 ratio) spun at 300 x g for 5 mins, ~2mls of homogenate was kept for analysis.

Supernatant was discarded. Pellet was resuspended in ~10mls of RPMI-1640.

Monocytes adhered to the plastic of the petri dish and were removed using a cell scraper. Opaque interface was removed. Spun at 300 x g for 5 mins, ~2mls of red cells were kept for analysis.

Cells were washed twice in PBS. Supernatants were discarded and the pellets were resuspended in either lysis buffer so as to prepare cell lysates or STE buffer for mitochondrial isolations.

Figure 2.4: Schematic illustration of the method used for the isolation of spleen cells.
carefully aspirated using a Pasteur pipet and discarded. The opaque interface was then carefully transferred with a Pasteur pipet to a fresh 15 ml centrifuge tube and centrifuged at 300 x g for 5 minutes. Whilst this sample was undergoing centrifugation, the red cell pellet was resuspended in RPMI-1640 (10% FCS, L-glutamine), labeled and aliquoted for western blot analysis. After centrifugation, the pellet containing lymphocytes and monocytes was resuspended in 2 mls of RPMI-1640 (10% FCS, L-glutamine) and gently poured into a petri dish. The monocyte/lymphocyte mixture was allowed stand at room temperature for 1 hour. In this time, the monocytes would adhere to the plastic of the petri dish whilst the lymphocytes would remain in suspension. After 1 hour at room temperature, the lymphocytes were aspirated off, using a Pasteur pipet, into a fresh centrifuge tube, labeled and aliquoted for western blot analysis. The monocytes were scraped off the base of the petri dish, using a cell scraper, and resuspended in RPMI-1640 (10% FCS, L-glutamine). Similarly, this monocyte fraction was labeled and aliquoted for western blot analysis. Prior to western blot analysis, the spleen cell homogenate, red cells, lymphocyte and monocyte fractions were centrifuged at 300 x g for 5 minutes, and washed twice in phosphate buffered saline (PBS) prior to either a lysis procedure or mitochondrial isolation.

[2.11] Isolation of Thymus and Spleen cell mitochondria

Following on from section 2.9 and 2.10, all spleen cell homogenate, red cells, monocytes and lymphocytes and thymus cells (thymocytes) fractions were spun at 300 x g for 5 minutes at 4°C. The supernatants were discarded and all cellular fractions were resuspended in ice-cold (0-4°C) STE buffer (250 mM Sucrose, 5 mM Trizma-base, 2 mM EGTA, pH 7.4). The cell suspensions were poured into a 2 ml Potter homogeniser tube to a final volume of about 1.5 mls. The cellular suspensions were then homogenised by hand, with 6 passes using a pestle of 0.12 inch (tight) clearance. The cell homogenates were centrifuged at 800 x g for 3 minutes at 4°C. The pellets were discarded and the supernatants were centrifuged at 12,000 x g for 10 minutes at 4°C yielding a “mitochondrial” pellet. The supernatants were discarded and the pellets were resuspended in STE buffer and re-centrifuged at 12,000 x g for 10 minutes at 4°C. The pellets were resuspended in buffer and centrifuged as above.
The resulting pellets containing the mitochondrial fraction were resuspended in STE buffer to the desired concentration and stored on ice or at –20°C, prior to analysis.

[2.12] Mitochondrial Protein Determination using the Markwell Assay

The amount of protein in the mitochondrial isolations was measured using a modification (Markwell et al., 1978) of the method of Lowry et al., (1951). The following solutions were used:

Markwell A: 2% (w/v) Sodium Carbonate  
0.4% (w/v) Sodium Hydroxide  
0.16% (w/v) Sodium Potassium Tartrate  
1% (w/v) SDS

Markwell B: 4% (w/v) Copper Sulphate (CuSO₄·5H₂O)

Markwell C: A 1 in 100 (v/v) dilution of Markwell B with Markwell A.

A standard protein solution of BSA was suitably diluted to 0-100 μg/ml to a total volume of 0.5 mls. Samples were measured using two dilutions in triplicate. An aliquot (1.5 mls) of Markwell C was added to BSA standards and unknown protein solutions. After mixing the solutions, they were left at room temperature for 15 minutes. This was followed by the addition of 0.15 ml of Folin-Ciocalteau reagent, diluted 1:1 (v/v) with distilled water. After mixing, the samples were incubated at room temperature for 45 minutes. The absorbance of each sample was measured spectrophotometrically at a wavelength of 650nm. The amount of protein present was determined by reference to a standard curve of known concentrations of BSA over the range of 0-100 μg/ml.

[2.13] SDS PAGE Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970), using a Mini-PROTEAN® 3 CELL (Bio-Rad) with a 5% stacking and a 12% resolving gel. Components of a 12% resolving gel are as follows: water (3.35 ml), 1.5 M Tris-HCl, pH 8.8 (2.5 ml), 10%
(w/v) SDS (100 μl), Protogel™ (ultra-pure 30% (w/v) acrylamide and 0.8% (v/v) bisacrylamide solution; National Diagnostics; 4.0 ml), TEMED (5 μl) and 10% (w/v) ammonium persulphate (50 μl). The stacking gel (5%) was prepared with water (6.1 ml), 0.5 M Tris-HCl, pH 6.8 (2.5 ml), 10% (w/v) SDS (100 μl), Protogel™ (ultra-pure 30% (w/v) acrylamide and 0.8% (v/v) bisacrylamide solution; National Diagnostics; 1.33 ml), TEMED (10 μl) and 10% (w/v) ammonium persulphate (50 μl).

Prestained molecular weight marker standards (provided by New England Biolabs) were used. The prestained markers used were *E.coli* MBP-β-galactosidase (175 kDa), *E.coli* MBP-paramyosin (83 kDa), bovine liver glutamic dehydrogenase (62 kDa), rabbit muscle aldolase (47.5 kDa), rabbit muscle triosephosphate isomerase (32.5 kDa), bovine milk β-lactoglobulin A (25 kDa), chicken egg white lysozyme (16.5 kDa) and bovine lung aprotinin (6.5 kDa).

The sample buffer was made up as 4 X strength. Components of the sample buffer are as follows: water (3.8 ml), 0.5 M Tris-HCl, pH 6.8 (1.0 ml), glycerol (0.8 ml), 10% (w/v) SDS (1.6 ml) and 1% (w/v) Bromophenol blue (0.4 ml). 5% β-mercaptoethanol (v/v) was added to the sample buffer immediately prior to use. The sample buffer was added to the mitochondrial protein so that the final concentration of sample buffer was 1 X. Once the sample buffer and mitochondrial protein were added together, the samples were vortexed briefly and boiled for 5 minutes on a heating block set to 100°C. The samples were then pulsed in a bench top centrifuge, loaded into separate wells and electrophoresed at a constant current (200 V) for 45 minutes, until the tracker dye reached the bottom of the resolving gel. A glycine-based running buffer (0.38 M glycine, 0.05 M Tris, 0.1% (w/v) SDS) was used for electrophoresis. Gels were then prepared for Western Blotting.

**[2.14] Western Blotting**

Following SDS-PAGE, resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-PSQ; Millipore). Transfer was achieved using a semi-dry transfer apparatus (Hoeffer) at 110 mA for 2 hours. Firstly, the stacking gel was removed and gels were rinsed briefly in semi-dry transfer buffer (0.192 M glycine, 0.025 M Tris-HCl, pH 8.3, 0.013 M SDS, 15%
(v/v) methanol) and carefully arranged in the semi-dry transfer apparatus as directed by the manufacturers. After transfer was complete, blotted proteins on the PVDF membrane were directly incubated in Ponceau S solution (0.25% (w/v) Ponceau S, in 3% (v/v) trichloroacetic acid) and washed gently with distilled water. The blot was then washed in phosphate-buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 11.5 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4). Blocking of the membrane was performed by incubating the blot in PWB (PBS containing 0.1% (w/v) Tween 20) containing 5% (w/v) Marvel milk powder at room temperature for 1 hour or overnight at 4°C. This blocking was followed by 3 X 10 minute washes using PWB.

Blots were then incubated in primary antibody (in PWB containing 5% (w/v) Marvel milk powder) overnight at 4°C or at room temperature for 1 hour containing a 1:1,000 dilution of an affinity-purified anti-UCP 1 and UCP 3 peptide antibody, a 1,1000 dilution of a commercial anti-UCP 1, UCP 2 and UCP 3 peptide antibody (Calbiochem), a 1:1,000 dilution of a peptide antibody to subunit III of cytochrome oxidase (COIII) and a 1:1,000 dilution of an antibody to the β-subunit of the F$_1$-ATP synthase (F$_1$β). Following this primary antibody incubation, the blots were washed for 3 X 10 minute in PWB. The blots were then incubated with a horse-radish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (1:10,000 dilution) in PWB containing 5% Marvel milk powder for 1 hour at room temperature. Following this, blots were further washed for 3 X 10 minute in PWB. Blots were developed using an enhanced chemiluminescence (ECL) detection system (Amersham-Pharmacia) for detecting horse-radish peroxidase labeled antibody, by means of the HRP catalyzed oxidation of luminol under alkaline conditions and the results were visualized by exposure to Kodak X-Omat LS film.

[2.15] Coomassie Blue R staining of SDS-PAGE gels

Following SDS-PAGE, the laemmli gels were stained using Coomassie blue R according to the method of Laemmli (1970) so as to ensure all the protein was transferred to the membranes successfully. Laemmli gels were stained using 0.5% (w/v) Coomassie Blue R in 50% (v/v) methanol, 10% (v/v) acetic acid for 30 minutes on a shaking platform. The Coomassie Blue R stain was then discarded and
the gels were destained on a shaking platform for 30 minutes using a 50% (v/v) methanol and 10% (v/v) acetic acid.

[2.16] Densitometry
Following Western blot analysis, the relative abundance to UCP 1, UCP 2 and UCP 3 was determined using densitometry. The band intensities of the exposed film were analysed using Scion Imaging Software.

[2.17] Antibodies
Peptide synthesis, conjugation of peptides to BSA and injection of rabbits was undertaken by Eurogentec. Polyclonal antibodies in the resulting rabbit anti-sera were affinity-purified on a protein A column. The UCP peptides based on rat sequence to which polyclonal antibodies were raised are as follows:

UCP 1: 145..... CHLHGKPRYTGYNA........158
UCP 2: 147..... QARAGGGRYYQSTVEA.....162
UCP 3: 141..... TGGERKYRGTMDAYRC.....156

Commercial anti-UCP 1 (145aa-159aa), anti-UCP 2 (144aa-157aa) and anti-UCP 3 (147aa-159aa) peptide antibodies were obtained from Calbiochem. The cytochrome-oxidase III (COIII) peptide antibody was generated by Eurogentec to the C-terminal end of the protein: H2N-MTHQTHAYHMV-COOH. The antibody to the β-subunit of the F1-ATP synthase was raised to that of *Neurospora crassa* and was a gift from Dr. Matt Harmey, Department of Botany, University College Dublin, Ireland.

[2.18] Affinity purification of Polyclonal antibodies using Protein A
The protein A column was prepared by swelling 0.5 g of protein A (Sigma) in 10 mls of 0.1 M Tris-HCl, pH 8 overnight at 4 °C. 0.5 g of protein A swells to a bed volume of 2 mls. The column was set by pouring Protein A into a 10 ml syringe barrel, previously plugged with glass wool. The column was allowed to flow by gravity to pack the column bed. The packed affinity resin was then equilibrated with 10 column volumes of 0.1 M Tris-HCl, pH 8. In the meantime, the pH of the
antibody serum was adjusted to pH 8.0 by adding 1/10th the volume of 1 M Tris-HCl, pH 8.0 (e.g. 0.05 ml of 0.1 M Tris-HCl, pH 8.0 was added to 0.5 ml of serum sample). The serum sample was then gently applied to the column, ensuring at all times not to disturb the bed surface. The column was then washed with 10 column volumes of 0.1 M Tris-HCl, pH 8.0. The column was further washed with 10 column volumes of 0.01 M Tris-HCl, pH 8.0. The bound immunoglobulin was eluted with 4 column volumes of 0.1 M glycine, pH 3.0. Before beginning elution, enough eppendorf tubes were set up to collect the entire elution volume as 0.5 ml fractions. 0.05 mls of neutralization buffer (1 M Tris-HCl, pH 8.0) was added to each tube prior to collection of elute. Following this, the elution buffer (0.1 M glycine, pH 3.0) was gently added to the top of the resin. 0.5 ml fractions were collected until the elution buffer has just entered the column bed. The immunoglobulin fractions were then quantified spectrophotometrically at 280nm using the formula: 1 OD (optical density) = 0.8 mg/ml. The immunoglobulin fraction consisting of the greatest protein concentration was aliquoted and stored at −20°C prior to western blot analysis. Once the sample had been eluted from the column, the affinity matrix was washed with 2 column volumes of 0.1 M glycine, pH 3.0. This was followed by re-equilibration of the column with at least 10 column volumes of 0.1 M Tris-HCl. These latter steps were performed to ensure the successful regeneration of the column for future use.

[2.19] Purification of UCP 1 from thymus mitochondria

UCP 1 was purified from thymus mitochondria using a modified protocol from Lin & Klingenberg, (1980). 8-10 mg of total thymus mitochondrial protein, suspended in STE buffer, (250 mM sucrose, 5 mM Trizma-base, 2 mM EGTA, pH 7.4) were centrifuged at 22,600 x g for 10 minutes at 4°C. The mitochondrial pellet was solubilised using a solution of 13% (v/v) octylpentaoctylethylene ether (Octyl-POE)/STE solution (total volume 471.2 µl) and incubated on ice for 10 minutes. The solubilised mitochondria were loaded onto hydroxypetite (HTP) column. The HTP column was prepared by soaking 0.34 grams of HTP in at least 10 mls of STE buffer, pH 7.4, at 4°C, for at least 6 hours prior to pouring it into a 5 ml BioRad column. The soaking solution was removed by centrifugation at 800 x g for 2 minutes at room temperature. The HTP column, containing the solubilised mitochondria, was
incubated at room temperature for 10 minutes (to denature the adenine nucleotide carrier) followed by a 25 minute incubation at 4°C. The column was then centrifuged at 800 x g for 2 minutes to remove the HTP elute. The protein concentration of this HTP elute was determined and the Octyl-POE detergent was removed using a Biobead (BioRad) column. The Biobead column was prepared by placing 2mls of Biobeads (previously suspended in UHP-H_2O), into a 2ml syringe barrel and allowed to flow by gravity to pack the column bed. The UHP-H_2O was removed from the Biobeads by centrifugation at 800 x g for 2 minutes. The Biobeads were then equilibrated in ~2 mls of STE buffer, pH 7.4, for 30 minutes prior to use. Biobeads were further centrifuged at 800 x g for 2 minutes to remove excess STE buffer, pH 7.4. The HTP elute was then loaded onto Biobeads, mixed gently, and incubated for two-hours at 4°C with slight agitation using a vortex. The Biobead column was then centrifuged at 800 x g for 2 min and the pure protein elute was collected. The protein concentration of this elute was determined. Approximately, 5-10 μg of protein was subjected to SDS-PAGE electrophoresis.

[2.20] Colloidal B Satin staining for SDS-PAGE gels

Following separation of the purified UCP 1 protein using SDS-PAGE, the UCP 1 protein band was stained using a Colloidal B Satin method, as previously described in the literature (Neuhoff et al., 1998).

Firstly, disposable gloves, previously soaked in ethanol were used at all times. All glassware was washed with ethanol and UHP-H_2O, and the staining procedure was performed in a fume hood so as to reduce the possible contamination of keratins. SDS-PAGE gels were agitated in fixative (50% (v/v) ethanol, 3% (v/v) ortho-phosphoric acid) for 2 days at room temperature (200 mls per gel). Following fixation, gels were washed for 3 X 30 minutes in UHP-H_2O (500 ml per gel). The gels were then allowed to equilibrate in Neuhoff’s solution (16% (w/v) ammonium sulphate, 25% (v/v) methanol, 5% (v/v) ortho-phosphoric acid; Neuhoff et al., 1988) with gentle agitation for 1 hour at room temperature. 1 gram of Coomasie Brilliant Blue G-250 (SERVA blue G; research grade) was sprinkled into the Neuhoff’s solution and staining was continued for 2-3 days at room temperature. Once the
highly stained single protein band at ~32.5 kDa was visible, the intact gel slice was transferred to a 20% (w/v) ammonium sulphate solution prior to sequence analysis.

[2.21] Dowex treatment of isolated mitochondria

Endogenous residual bound nucleotides were removed from isolated mitochondria using an anion exchanger (Dowex 21K) by the procedure described by Huang & Klingenberg (1995). Isolated mitochondria at a concentration of 2 mg/ml in a buffer containing 250 mM sucrose, 20 mM Hepes, 1 mM EDTA, pH 8.0, were shaken with Dowex (120 mg/mg protein) at room temperature for 1 hour.

[2.22] GDP Binding Assay

Measurement of binding of [³H] GDP was performed by a modification of the procedure described by Scarpace et al., (1991) using glass fibre filters that had been pre-soaked in a ligand binding assay buffer (100 mM Sucrose, 1.2 mM K-EDTA, 12.2 mM Choline Chloride, 40 mM TES, pH 7.1).

Mitochondria (50µg) were incubated with various concentrations (0.1 µM-6.0 µM) of [³H] GDP (11.0 & 12.5 Ci/mmol) in a ligand binding assay buffer (100 mM Sucrose, 1.2 mM K-EDTA, 12.2 mM Choline Chloride, 40 mM TES, pH 7.1) with the addition of 0.1 mg/ml fatty acid free BSA, 2µM rotenone and 30µM atractyloside in a total volume of 0.25 ml at 37°C. [¹⁴C] Sucrose (250 µCi/ml) was included in the incubations to calculate the volume of medium trapped on the filter. Non-specific binding was determined in the presence of 1.5 mM unlabelled ligand (GDP). All samples were incubated in triplicate for 15 minutes. The incubation mixtures were diluted with 3 ml of ligand binding assay buffer (left at room temperature) and filtered immediately through Whatman glass fibre filters (GF/B, 2.5cm) that had been pre-soaked in assay buffer. The filters were then washed with a further 2 X 3mls of ligand binding assay buffer. The total time taken for filtration and washing was less than 10 seconds. Filters were allowed to dry at room temperature before being placed in scintillation vials. Ecoscint™ scintillation fluid (10 mls) was then added carefully to the dried filters for fluid scintillation counting. Filters in scintillation fluid were vortexed vigourously and left at room temperature.
overnight to ensure thorough dissolution of the filters. Samples were then counted as described in section 2.25.

[2.23] Liquid Scintillation Counting

The \(^{3}\text{H}\) isotope and \(^{14}\text{C}\) isotope was measured using a Packard TriCarb 1500 and 2100 scintillation spectrophotometer. The cocktail used was the commercially available scintillant, Ecoscint\textsuperscript{TM}, for aqueous samples. The average counting efficiency for \(^{3}\text{H}\) counting using this instrument was calculated to be 45\% and for \(^{14}\text{C}\) sucrose was calculated to be 96 \% based on a quench correction curve relating counting efficiency to the spectral index of the external standard.

[2.24] Analysis of Saturable Binding Data

Binding values were obtained as disintegrations per minute (dpm) and converted to pmoles of bound radioligand per milligram of mitochondrial protein using 100\% counting efficiency and specific radioactivity values. The mean values and the standard error of the mean values (S.E.M) were calculated for total and non-specific binding using triplicate samples and the following formula was employed to obtain standard error of the mean for specific binding values:

\[
\text{S.E.M} = \sqrt{(E_1)^2 + (E_2)^2}
\]

Where \(E_1 = \text{S.E.M for total binding and } E_2 = \text{S.E.M for non-specific binding.}

Specific binding was calculated from the difference between total and non-specific binding.

The Michaelis dissociation constant (\(K_D\)) and the maximal binding capacity (\(B_{\text{MAX}}\)), were obtained by fitting mean values for specific binding sites on the y-axis and free radioligand concentrations used on the x-axis using the computer program “Sigma Plot”, version 5.

This program estimates \(K_D\) and \(B_{\text{MAX}}\) parameters, after the data are fitted directly to an equation relating the concentration of the radioligand-receptor complex [Bound] and the free radioligand concentration [Free ligand]:

53
[Bound] = B_{MAX} \times [\text{Free ligand}] / ([\text{Free ligand}] + K_D)

which describes a rectangular hyperbola (and is mathematically equivalent to the Michaelis-Menten equation of enzyme kinetics). The data was fitted by non-weighted, non-linear least squares regression. The data points are represented by a line of best fit.


Dioxygen uptake (probe for electron flow) by suspensions of isolated mitochondria can be studied using an oxygen electrode. The electrode (Clarke-type) consists essentially of a silver/silver chloride reference half-cell, joined to a platinum/O_2 cathode by a saturated KCl bridge. The electrode compartment is isolated from the reaction chamber (in which the mitochondria and their substrates or inhibitors interact) by a thin teflon membrane. The membrane is permeable to dioxygen and allows this gas to reach the cathode, where it is electrolytically reduced. The reduction allows a current to flow and this is converted to a potential and recorded on a chart recorder. The trace is thus a measure of the dioxygen content of the reaction mixture and both the extent and rate of dioxygen uptake by mitochondria maybe measured.

The mitochondrial suspension in the reaction chamber was stirred using a magnetic stirrer and thermostatically maintained at 37°C.

[2.26] Fatty Acid Activation Assay

The sensitivity of respiration by non-phosphorylating mitochondria to fatty acids and purine nucleotides was evaluated using the procedure essentially as described by González-Barroso et al., (1998). Mitochondria (1mg/ml) were incubated at 37°C in medium containing 120 mM KCl, 5 mM Hepes, 1 mM EGTA, pH 7.4, 16μM fatty-acid free BSA, 5 μM rotenone and 1 μg/ml oligomycin, 5μM atractyloside. Non-phosphorylating (state 4) oxygen consumption rate were measured as the steady-state rate achieved on addition of 7.5 mM succinate (succinate-KOH, pH, 7.4). The sensitivity of this state 4 oxygen consumption rate to
GDP (1mM) was then determined. The sensitivity of the resulting oxygen consumption rate to palmitate (in ethanol) (64 μM)(~40 nM free) was then determined. Finally, the mitochondrial uncoupler cyanide-\( p \)-trifluoromethoxyphenylhydrazone (40 nM FCCP) was added to the chamber to determine the maximum oxygen consumption rate attainable due to uncoupling. The Clark oxygen electrode was calibrated according to the procedure of Reynafarje et al. (1985) assuming 406 nmoles O dissolved in 1ml of incubation medium at 37°C.

[2.27] **Statistical Analysis**

Data are presented as mean ± S.E.M. The mean values were compared using an unpaired (two-tailed) Student's T-test. A probability P-value is thus obtained indicating whether the means are significant (P<0.05).
Chapter 3:

UCP 1, UCP 2 and UCP 3 mRNA expression

using RT-PCR and PCR analysis:
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[3.1] Introduction

Traditionally, expression of eukaryotic mRNA’s have been analysed by procedures such as Northern blotting. Northern blot analysis, using radiolabelled probes, remains the standard for detection and quantitation of mRNA levels despite the advent of more sensitive techniques. Northern blot analysis presents several advantages over other RNA expression techniques. The most compelling of these is that it is the easiest method for determining transcript size, and for identifying alternatively spliced transcripts and multigene family members. Northern blotting is semi-quantitative, relatively accurate and can be used to directly compare the relative size and abundance of a given mRNA between all samples on a blot. Despite these advantages, there are limitations associated with Northern blot analysis. Firstly, if RNA samples are even slightly degraded, the quality of the data and the ability to quantitate expression are severely compromised. Secondly, Northern blot analysis is, in general, the least sensitive technique for detecting low abundance mRNA expression levels compared to nuclease protection assays, in situ hybridization and reverse-transcriptase polymerase chain reaction (RT-PCR). Northern blotting requires total RNA in excess of several micrograms (µg), even when examining gene transcripts expressed at high levels. Typically, RNA analysed by Northern blotting must be further enriched for mRNA by oligo (dT) cellulose chromatography, and is then only sensitive from 5-10 µg poly (A) + mRNA. Northern blotting techniques also require transfer and hybridization of each mRNA and the use of specifically labeled radioactive probes. Another limitation of Northern blotting has been the difficulty associated with multiple probe analysis. To detect more than one message, it is usually necessary to strip the initial probe before hybridizing with a second probe. This process can be time consuming and problematic.

Despite these disadvantages, Northern blot analysis, using radiolabelled nucleotides, has been key in identifying possible locations and functions for UCP 1, UCP 2 and UCP 3 (Boss et al., 1997; Gong et al., 1997; Larrouy et al., 1997; Masaki
et al., 1997; Nègre-Salvayre et al., 1997; Boss et al., 1998; Hodný et al., 1998; Yoshitomi et al., 1998; Giacobino et al., 1999; Matthias et al., 1999; Richard et al., 1999; Ricquier & Bouillaud, 2000; Pecqueur et al., 2001; Pedraza et al., 2001; Krauss et al., 2002; Horvath et al., 2003). Northern blot analysis has shown that UCP 1 mRNA is expressed in brown adipose tissue (BAT), where it provides the basis for nonshivering thermogenesis in mammals (Cannon et al., 1982; Ricquier & Bouillaud, 1986; Boss et al., 1997; Nègre-Salvayre et al., 1997; Boss et al., 1998; Giacobino et al., 1999; Richard et al., 1999; Ricquier & Bouillaud, 2000; Pedraza et al., 2001). Northern blot analysis has also shown that UCP 2 mRNA is present in a panoply of tissues of mice, rats and humans with highest levels being found in thymus, spleen, intestine, lung, brain, kidney, BAT, white adipose tissue (WAT), fetal liver and in the Kupffer cells of the mature liver (Fluery et al., 1997; Larrouy et al., 1997; Nègre-Salvayre et al., 1997; Gong et al., 1997; Boss et al., 1998; Hodný et al., 1998; Ricquier & Bouillaud, 2000; Pecqueur et al., 2001; Pedraza et al., 2001; Krauss et al., 2002; Horvath et al., 2003). Similarly, Northern blot analysis shows that UCP 3 transcript has only ever been detected in BAT and skeletal muscle with transcript having particular prominence in tensor fascia latae (fast-twitch, glycolytic), tibalis anterior (fast-twitch, oxidative/glycolytic) and gastrocnemius (mixed) muscles and lower levels in the soleus muscle (slow-twitch, oxidative) (Boss et al., 1997 & 1998a; Vidal-Puig et al., 1997; Cadenas et al., 1999; Giacobino et al., 1999; Pedraza et al., 2001).

The use of digoxigenin (DIG) labeled probes instead of 32P-labeled probes in Northern blotting, have been even less successful in detecting UCP’s. DIG-labeled probes have detected UCP 3 mRNA in COS cells expressing UCP 3 (O. Cunningham & R.K. Porter, unpublished data) and in fetal rat brown adipocytes (Tureul et al., 2000). However, these DIG-labeled probes are not sensitive enough to detect constitutively expressed UCP 1, UCP 2 and UCP 3 mRNA in tissues and cells, that have been previously shown to express UCP’s using 32P-labeled probes.

Reverse-transcription, followed by the amplification of the polymerase chain reaction (PCR) technique, provides another method of detecting the presence of mRNA (Saiki et al., 1985). Such a PCR-based method has been termed reverse-transcriptase-PCR (RT-PCR) (Larrick & Siebert, 1995). RT-PCR has revolutionized
the study of gene expression. It is now theoretically possible to detect the RNA transcript of any known gene, regardless of the scarcity of the starting material or relative abundance of the specific mRNA. The advantages of the RT-PCR technique include its versatility, sensitivity and rapid turn-around time (Larrick & Seibert, 1995; Dean et al., 2002). Of all the methods available, RT-PCR is the most sensitive method of RNA detection available. RT-PCR detects the expression of specific genes, which have low abundance RNA or are present in limiting amounts of tissue (He et al., 1995). Data from RT-PCR can be obtained on < 1 ng of total RNA, which eliminates the poly (A) + mRNA purification step of other Northern blotting techniques. RT-PCR can detect changes in expression of multiple mRNA’s simultaneously and further detect gene transcripts from small amounts of RNA (Larrick & Siebert, 1995). RT-PCR, like Northern blotting, is semi-quantitative. RT-PCR has been shown to be 1,000-10,000 fold more sensitive than the traditional Northern blotting techniques in detecting gene expression (He et al., 1995).

Additionally, RT-PCR does not require the use of radionucleotides and can be performed in a few hours, compared to the overnight incubation required when working with $^{32}$P-labeled nucleotide probes. The exquisite sensitivity of RT-PCR over Northern blotting has been demonstrated by several research groups in the literature (Lee et al., 1999; Jekabson et al., 2001; Chelly et al., 1988; Fuqua et al., 1990; Murphy et al., 1990; Gaudette et al., 1991). Lee et al. (1999) has shown that IL-6 mRNA was not detected in macrophages from ob/ob mice using Northern blot analysis, but IL-6 mRNA was detected in macrophages from ob/ob mice using RT-PCR analysis. Similarly, Jekabson et al. (2001) showed that UCP 2 mRNA was not detected in gastrocnemius, soleus and tibialis anterior muscle groups isolated from lean Zucker rats using Northern blot analysis, whereas UCP 2 mRNA was detected in these muscle groups using RT-PCR analysis. The dystrophin gene, defective in patients with muscular dystrophy, is expressed at very low levels (representing only 0.01-0.001% of total muscle mRNA), making it difficult to study by conventional methods, such as Northern blotting. RT-PCR was successfully used by Chelly et al. (1988) to study levels of dystrophin mRNA in clinical samples. The poorly expressed multi-drug resistance gene mdr-1 has also been studied by RT-PCR, whereas conventional methods were unsuccessful at finding transcripts (Fuqua et al.,
1990; Murphy et al., 1990). In some experimental models, genes may be expressed at moderate to high levels, but in tissues of minute size such as early mouse embryos that RT-PCR has proved to be a valuable tool (Gaudette et al., 1991). These authors thus demonstrate that RT-PCR analysis is a more sensitive technique than Northern blot analysis for demonstrating the presence of low-abundance mRNA's.

RT-PCR has been successful in detecting mRNA expression of UCP's. Using RT-PCR analysis, Oberkofler et al. (1997) showed that UCP 1 mRNA is expressed in adipose tissue of obese and non-obese humans. Larrouy et al. (1997) demonstrated that UCP 1 mRNA was not detected in liver from adult rats. However they did show that UCP 2 mRNA was expressed preferentially in the Kupffer cells of the adult liver but not in hepatocytes or parenchyma cells. Cabrero et al. (1999) showed that etomoxir induced a 3.6-fold increase in UCP 3 in a primary culture of rat preadipocytes, but had no effect on UCP 2 mRNA levels. Similarly, Cabrero et al. (2000 & 2001) went onto show that UCP 2 and UCP 3 mRNA levels were down-regulated by thiazolidinediones in C2C12 myotubes, whereas UCP 3 mRNA levels were upregulated in C2C12 myotubes after etomoxir treatment.

Three decades of research suggest that UCP 1 is exclusive to BAT, where it appears to function as a true thermogenic protein (Bouillaud et al., 1985). More recently, homologues of UCP 1, UCP 2 and UCP 3, have been discovered (Fluery et al., 1997; Boss et al., 1997). As a prelude to elucidating their physiological function, we investigated the pattern of UCP mRNA expression in certain tissues. One of the aims of this study was to have a quick and easy, yet sensitive and distinguishing (not necessarily quantitative) assay for detecting UCP mRNA. The RT-PCR/PCR methodology was chosen with the knowledge that RT-PCR fulfilled these criteria, had greater sensitivity than Northern blotting and that RT-PCR has previously been shown to be successful in detecting UCP mRNA expression.

The UCP 1, UCP 2 and UCP 3 primers, employed in this RT-PCR study, were adapted from Emilsson et al. (1998). GAPDH gene was chosen as the endogenous internal house-keeping gene as it is expressed constitutively in every cell (Glare et al., 2002). The GAPDH gene contains introns so it is possible to design primers that bridge an exon-intron-extron boundary in order to amplify RNA products and not genomic DNA. GAPDH primer design was adapted from Tokunga
et al. (1987) and the same GAPDH primers has been previously employed by McElligott, (1999, Ph.D thesis). Similarly, with UCP 1, 2 and 3 primers, the sense primers were designed bridging an exon-intron-exon boundary, ensuring that no genomic DNA would be amplified. The PCR primers used are between 18-22 nucleotides long and have an A/T content about equal to the G/C content, so that the optimal annealing temperatures of both forward and reverse primers are similar (Table 2.1). The RT-PCR assay employed here, although semi-quantitative, offers the advantage of high sensitivity, lack of cross reactivity and rapid analysis of expression of different genes simultaneously.

Cumulative data from previous studies have shown that UCP 1, UCP 2 and UCP 3 mRNA are expressed in rat BAT (Fluery et al., 1997; Boss et al., 1997; Gong et al., 1997; Boss et al., 1998; Matthias et al., 1999; Pedraza et al., 2001; Ricquier & Bouillaud, 2000). Collation of data from previous studies has also shown that UCP 1 (Ricquier & Bouillaud, 1986 & 2000; Richard et al., 1999; Larrouy et al., 1997; Hodný et al., 1998), UCP 2 (Boss et al., 1997) and UCP 3 (Boss et al., 1997; Richard et al., 1999; Emilsson et al., 1998; Hodný et al., 1998) transcripts are not expressed in rat liver. Therefore, in this study, we sought to use rat BAT and liver as positive and negative control tissues, respectively, for our UCP primers. We also became interested in investigating UCP expression in the thymus and spleen as evidence for a role for UCP 2 in thymocyte proton leak came from the fact that ablation of the UCP 2 gene decreases proton leak in in situ mitochondria of isolated mouse thymocytes (Krauss et al., 2002). UCP 2 mRNA has been previously shown to be expressed in human and rat thymus and human and rat spleen (Gong et al., 1997; Nègre-Salvayre et al., 1997; Jekabsons et al., 2001; Horvath et al., 2003; Pecqueur et al., 2001; Richard et al., 1999; Ricquier & Bouillaud, 2000). In our attempts to investigate the role of UCP’s in mediating proton leak, we sought to confirm the observation that UCP 2 is expressed in thymus and spleen at the level of transcript, using the highly sensitive RT-PCR methodology, and to determine whether UCP 2 is the sole UCP present in these tissues.
Results:

UCP gene expression using RT-PCR and PCR analysis:

The use of high-quality RNA is critical for the success of RT-PCR analysis. The RNA must not be degraded by ribonucleases, as determined by the intactness of ribosomal RNA (rRNA) bands. The intactness of the total RNA preparations was examined by denaturing agarose gel electrophoresis (Figure 3.2.1). 5μg of total RNA isolated from rat BAT (Lane B), thymus (Lane T) and liver (Lane L) were electrophoresed on a 1% formaldehyde denaturing agarose gel (Figure 3.2.1), as described in section 2.4. The 28S and 18S ribosomal RNA (rRNA’s) bands are clearly indicated. The 28S and 18S ribosomal RNA (rRNA) bands show an approximate 2:1 ratio of band intensities indicating that the total RNA is intact and that no significant RNA degradation has occurred.

The initial RT-PCR/PCR experimental and amplification conditions for GAPDH, were optimized in our laboratory by Higgins et al. (2001). Higgins et al. (2001) shows amplification of ubiquitously expressed GAPDH in C2C12 myotubes cDNA was linear between 21-35 cycles, while the yield reached a plateau after 35 cycles. In all RT-PCR and PCR experiments used in this study, care was taken to ensure measurements were in the linear range of amplification. In nearly all cases, specific single amplification products were generated with no additional bands. Similar assay conditions have been used previously to compare mRNA expression of UCP 1, 2 and 3 (Emilsson et al., 1998; Yoshitomi et al., 1998).

Figure 3.2.2 (A), (B) and (C) shows the expression of UCP 1 (Lane A), UCP 2 (Lane B) and UCP 3 (Lane C) mRNA in rat BAT, respectively, using RT-PCR analysis. Figure 3.2.2 (D) shows the detection of the house-keeping gene GAPDH (Lane D) in rat BAT. The RT-PCR methodology distinguishes between transcripts UCP 1 (399bp) (A), UCP 2 (302bp) (B), UCP 3 (364bp) (C) and GAPDH (444bp) (D) in rat BAT. For figures 3.2.2 (A), (B), (C) and (D), an RT control reaction (Lane R) and PCR control reaction (Lane P) yielded no PCR products. The RT control reaction incorporated DEPC-H2O in place of a total RNA template whilst
Figure 3.2.1: Analysis of total RNA integrity using denaturing agarose gel electrophoresis.

Total RNA was extracted using a TRI REAGENT™ from rat BAT (B), Thymus (T) and Liver (L) (as described in Section 2.3). Total RNA was quantified using $A_{260}/A_{280}$ measurements. 5 μg of rat BAT (B), thymus (T) and liver (L) total RNA were electrophoresed on a 1% formaldehyde denaturing agarose gel incorporating ethidium bromide (EtBr) at 60V for 3 h and visualised under a u.v transilluminator as described in Section 2.4. The 28S and 18S ribosomal RNA’s exhibit a near 2:1 ratio of ethidium bromide staining, indicating that no significant RNA degradation has occurred. Positions of the 28S, 18S ribosomal RNA are indicated. M corresponds to a 1 kb DNA ladder.
Figure 3.2.2: RT-PCR analysis showing expression of UCP 1, UCP 2, UCP 3 and GAPDH in rat brown adipose tissue (BAT).

Total RNA was isolated from rat BAT using a TRI REAGENT™. 1μg of total RNA was reverse transcribed to cDNA using AMV-reverse transcriptase and random primers. 3μl of this RT-reaction was used as a template for PCR. PCR amplification conditions entailed an initial denaturation of 5 minutes at 95°C followed by 35 cycles of 45 seconds at 95°C, 45 seconds at 55°C and 2 minutes at 72°C with a final extension for 10 minutes at 72°C using Taq DNA Polymerase and primers specific for UCP 1 (399bp), UCP 2 (302bp), UCP 3 (364bp) and GAPDH (444bp) transcripts. 10μl of each PCR reaction was analysed on a 1% agarose gel incorporating ethidium bromide and visualised under a u.v transilluminator (Sections 2.3, 2.5 and 2.6).

Lane M: 1 Kb DNA molecular markers; Lane R: RT-PCR control reaction (no total RNA template), Lane P: PCR control reaction (no cDNA template); Lane A, B, C and D: RT-PCR products using primers specific to UCP 1 (399bp), UCP 2 (302bp), UCP 3 (364bp) and GAPDH (444bp) transcripts respectively.
the PCR control reaction included DEPC-H\textsubscript{2}O, in lieu of a cDNA template. As no PCR products were observed using these controls, it is safe to conclude that the PCR products observed in figure 3.2.2 (A), (B), (C) and (D) are solely UCP 1, UCP 2, UCP 3 and GAPDH respectively.

Figure 3.2.3 (A), (B) and (C) demonstrates the application of the RT-PCR to liver using primers for UCP 1 (399bp), UCP 2 (302bp) and UCP 3 (364bp). Figure 3.2.3 (A), (B) and (C) shows the lack of expression of UCP 1 (Lane A), UCP 2 (Lane B) and UCP 3 (Lane C) mRNA in rat liver. Figure 3.2.3 (D) shows the expression of the internal control gene GAPDH (Lane D) in rat liver. For figures 3.2.3 (A), (B), (C) and (D), an RT control reaction (Lane R) and PCR control reaction (Lane P) yielded no PCR products. The RT control reaction incorporated DEPC-H\textsubscript{2}O in lieu of a total RNA template whilst the PCR control reaction included DEPC-H\textsubscript{2}O, in place of a cDNA template.

Figure 3.2.4 (A) shows the expression of UCP 1 (Lane A) mRNA in rat thymus (novel result). Figure 3.2.4 (B) detects UCP 2 (Lane B) mRNA expression in rat thymus. Figures 3.2.4 (C) shows the expression of the endogenous house-keeping gene, GAPDH (Lane D) in rat thymus. The RT-PCR methodology distinguishes between UCP 1 (399bp) (A), UCP 2 (302bp) (B) and GAPDH (444bp) (C) transcripts in rat thymus. In figures 3.2.4 (A), (B) and (C), an RT control reaction (Lane R) and PCR control reaction (Lane P) yielded no PCR products. The RT control reaction included DEPC-H\textsubscript{2}O in place of a total RNA template whilst the PCR control reaction incorporated DEPC-H\textsubscript{2}O, in lieu of a cDNA template. The expression of UCP 3 transcript in rat thymus was not investigated.

Figure 3.2.5 (A) shows the detection of UCP 2 (Lane B) transcript manifest in an adult human thymus cDNA library. Figure 3.2.5 (B) shows the expression of UCP 3 (Lane C) mRNA in an adult human thymus cDNA library (novel result). Figure 3.2.5 (C) shows the expression of the ubiquitous housekeeping GAPDH transcript (Lane D) in the adult human thymus cDNA library. The PCR methodology distinguishes between transcripts UCP 2 (302bp) (A), UCP 3 (364bp) (B) and
Figure 3.2.3: RT-PCR analysis showing lack of expression of UCP 1, UCP 2 and UCP 3 but shows subsequent detection of GAPDH in rat liver.

Total RNA was isolated from rat liver using a TRI REAGENT™. 1μg of total RNA was reverse transcribed to cDNA using AMV-reverse transcriptase and random primers. 3μl of this RT-reaction was used as a template for PCR. PCR amplification conditions entailed an initial denaturation of 5 minutes at 95°C followed by 35 cycles of 45 seconds at 95°C, 45 seconds at 55°C and 2 minutes at 72°C with a final extension for 10 minutes at 72°C using Taq DNA Polymerase and primers specific for UCP 1 (399bp), UCP 2 (302bp), UCP 3 (364bp) and GAPDH (444bp) transcripts. 10μl of each PCR reaction was analysed on a 1% agarose gel incorporating ethidium bromide and visualised under a u.v transilluminator (Sections 2.3, 2.5 and 2.6).

Lane M: 1Kb DNA molecular markers; Lane R: RT-PCR control reaction (no total RNA template), Lane P: PCR control reaction (no cDNA template); Lane A, B, C and D: RT-PCR products using primers specific to UCP 1 (399bp), UCP 2 (302bp), UCP 3 (364bp) and GAPDH (444bp) transcripts respectively.
Figure 3.2.4: RT-PCR analysis showing expression of UCP 1, UCP 2 and GAPDH in rat thymus.

Total RNA was isolated from rat Thymus using a TRI REAGENT™. 1μg of total RNA was reverse transcribed to cDNA using AMV-reverse transcriptase and random primers. 3μl of this RT-reaction was used as a template for PCR. PCR amplification conditions entailed an initial denaturation of 5 minutes at 95°C followed by 35 cycles of 45 seconds at 95°C, 45 seconds at 55°C and 2 minutes at 72°C with a final extension for 10 minutes at 72°C using Taq DNA Polymerase and primers specific for UCP 1 (399bp), UCP 2 (302bp) and GAPDH (444bp) transcripts. 10μl of each PCR reaction was analysed on a 1% agarose gel incorporating ethidium bromide and visualised under a u.v. transilluminator (Sections 2.3, 2.5 and 2.6).

Lane M: 1Kb DNA molecular markers; Lane R: RT-PCR control reaction (no total RNA template); Lane P: PCR control reaction (no cDNA template); Lane A, B and D: RT-PCR products using primers specific to UCP 1 (399bp), UCP 2 (302bp) and GAPDH (444bp) transcripts respectively.
Figure 3.2.5: PCR analysis showing expression of UCP 2, UCP 3 and GAPDH in a human thymus cDNA library.

-50ng of human thymus cDNA was subjected to PCR amplification. PCR amplification conditions entailed an initial denaturation of 5 minutes at 95°C followed by 35 cycles of 45 seconds at 95°C, 45 seconds at 55°C and 2 minutes at 72°C with a final extension for 10 minutes at 72°C using Taq DNA Polymerase and primers specific for UCP 2 (302bp), UCP 3 (364bp) and GAPDH (444bp) transcripts. 10μl of each PCR reaction was analysed on a 1% agarose gel incorporating ethidium bromide and visualised under a u.v transilluminator (Section 2.6).

Lane M: 1Kb DNA molecular markers; Lane P: PCR control reaction (no cDNA template); Lane B, C and D: PCR products using primers specific to UCP 2 (302bp), UCP 3 (364bp) and GAPDH (444bp) transcripts respectively.
GAPDH (444bp) (C). For figures 3.2.5 (A), (B) and (C), a PCR control reaction (Lane P) incorporating DEPC-H$_2$O in lieu of a cDNA template yielded no PCR products. Preliminary results indicate that no UCP 1 transcript is expressed in an adult human cDNA library (results not shown).

Figure 3.2.6 (A) shows no detection of UCP 1 (Lane A) mRNA in an adult human spleen cDNA library. Figure 3.2.6 (B) shows the expression of UCP 2 (Lane B) transcript in an adult human spleen cDNA library. Figure 3.2.6 (C) shows the detection of UCP 3 (Lane C) transcript in an adult human spleen cDNA library (novel result). Figure 3.2.6 (D) shows the constitutive expression of GAPDH (Lane D) in the human spleen cDNA library. The PCR methodology distinguishes between UCP 2 (302bp) (B), UCP 3 (364bp) (C) and GAPDH (444bp) (D) transcripts in the adult human spleen cDNA library. For figures 3.2.6 (A), (B), (C) and (D), a PCR control reaction (Lane P) incorporating DEPC-H$_2$O in lieu of a cDNA template yielded no PCR product.

Table 3.2 summarizes the results showing expression patterns of UCP 1, UCP 2, UCP 3 and GAPDH mRNA in the various tissues analyzed. Table 3.2 shows the detection of UCP 1, UCP 2, UCP 3 and GAPDH transcripts in rat BAT. UCP 1, UCP 2 and UCP 3 mRNA expression was not detected in rat liver, whereas GAPDH transcript was detected in rat liver. UCP 1 (novel result), UCP 2 and GAPDH mRNA were expressed in rat thymus. UCP 3 mRNA expression in rat thymus was not determined (ND). UCP 2, UCP 3 (novel result) and GAPDH transcripts were detected in both adult human thymus and spleen cDNA libraries. UCP 1 mRNA expression in an adult human thymus cDNA library was not satisfactorily determined (ND). No UCP 1 transcript was detected in an adult human spleen cDNA library.
Figure 3.2.6: PCR analysis showing expression of UCP 1, UCP 2, UCP 3 and GAPDH in a human spleen cDNA library.

~20ng of human spleen cDNA was subjected to PCR amplification. PCR amplification conditions entailed an initial denaturation of 5 minutes at 95°C followed by 35 cycles of 45 seconds at 95°C, 45 seconds at 55°C and 2 minutes at 72°C with a final extension for 10 minutes at 72°C using Taq DNA Polymerase and primers specific for UCP 1 (399bp), UCP 2 (302bp), UCP 3 (364bp) and GAPDH (444bp) transcripts. 10µl of each PCR reaction was analysed on a 1% agarose gel incorporating ethidium bromide and visualised under a u.v transilluminator (Section 2.6).

Lane M: 1Kb DNA molecular markers; Lane P: PCR control reaction (no cDNA template); Lane A, B, C and D: PCR products using primers specific to UCP 1 (399bp), UCP 2 (302bp), UCP 3 (364bp) and GAPDH (444bp) transcripts respectively.
Table 3.2: Summary of UCP 1, UCP 2, UCP 3 and GAPDH mRNA expression patterns in the various tissues analysed.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>UCP-1</th>
<th>UCP-2</th>
<th>UCP-3</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat BAT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat Liver</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Rat Thymus</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Human Thymus</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human Spleen</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Detection of transcripts is indicated by a “+” sign. No detection is indicated by a “−” sign. ND indicates the expression was not determined.
[3.3] Discussion

RT-PCR has never previously been used to detect basal expression levels of UCP’s in rat BAT. Using RT-PCR, with primers specific for detecting UCP 1, UCP 2 and UCP 3, we were able to confirm detection and distinguish between UCP 1, UCP 2 and UCP 3 transcripts in rat BAT (Figure 3.2.2). As BAT contains all three UCP’s, it acts as a clear positive control tissue for the RT-PCR methodology employed in this study. The detection of UCP 1, UCP 2 and UCP 3 mRNA in rat BAT confirms the results of several authors in the literature. Northern blot analysis has previously shown the expression of UCP 1 (Bouillaud et al., 1985; Nègre-Salvayre et al., 1997; Boss et al., 1998; Giacobino et al., 1999; Masaki et al., 1999; Gong et al., 1997), UCP 2 (Nègre-Salvayre et al., 1997; Boss et al., 1997; Fluery et al., 1997) and UCP 3 (Boss et al., 1997 &1998) transcripts in rat BAT.

Unlike rat BAT, rat liver showed no constitutive UCP mRNA expression, as determined by RT-PCR analysis (Figure 3.2.3). The lack of UCP’s in rat liver is a confirmatory result and thus acts as a negative control tissue for the RT-PCR methodology used in this study. Our results are consistent with several other authors in the literature. Larrouy et al. (1997), using RT-PCR analysis, showed the lack of UCP 1 mRNA expression in rat liver. Other research laboratories have shown, using northern blot analysis, the lack of UCP 1 (Bouillaud et al., 1985; Larrouy et al., 1997), UCP 2 (Boss et al., 1997) and UCP 3 (Gong et al., 1997) mRNA expression in liver of normal rats.

To date, most advances on determining physiological functions for UCP homologues have been associated with UCP 2. Krauss et al. (2002) have generated evidence for a role of UCP 2 mediating proton leak in intact thymocytes. In our endeavours to further investigate this proton leak, we sought to determine whether other UCP’s were also associated with the thymus. Our results, using RT-PCR analysis, shows detection of UCP 1 transcript in rat thymus (Figure 3.2.4). This is a novel, unexpected and exciting finding. It contrasts with the observation of Nègre-Salvayre et al. (1997), who were unable to detect UCP 1 mRNA in rat thymus using Northern blot analysis. This lack of UCP 1 mRNA detection in rat thymus, by
Nègre-Salvayre et al. (1997), suggests that UCP 1 mRNA exists in low abundance in the rat thymus (less than several μg’s) which would explain why UCP 1 mRNA in rat thymus could not be detected by Northern blotting. This is the first time UCP 1 has been detected outside BAT in rats. To find UCP 1 in thymus is unexpected as one might only expect to find UCP 1 in “thermogenic” tissues. As already mentioned, researchers have investigated its expression in other heat producing tissues such as skeletal muscle, brain and liver but no UCP 1 was detected. This study is also the first to show that UCP 1 is associated with cells/tissues of the immune system and suggests a new role for UCP 1 in the thymus that is not associated with thermogenesis. In contrast to rat thymus, our preliminary data indicates that UCP 1 mRNA was not expressed in an adult human thymus cDNA library (results not shown). In humans, BAT is found only in the newborn, and its activity decreases during infancy with only diminutive amounts ever found in the adult (Boss et al., 1997), except for patients with phaeochromocytoma (chromaffin tumors) (Néchad et al., 1986). Similarly, the thymus is very active early in life but activity decreases in adulthood (Ritter & Crispe, 1992). One might therefore surmise that UCP 1 is only associated in the developmental stage of the thymus and therefore its expression would diminish with age. If this is true, one might expect UCP 1 to exist in the thymus of a younger person. Future research will determine whether this is so.

Our study, using RT-PCR/PCR analysis, also shows the expression of UCP 2 mRNA in rat and human thymus (Figure 3.2.4 & 3.2.5). These results compliment several other reports in the literature, who showed using Northern blot analysis, that UCP 2 transcript is expressed in rat (Nègre-Salvayre et al., 1997) and human thymus (Gong et al., 1997).

The presence of UCP 3 in rat thymus was not determined in this study but we do show the novel finding that UCP 3 transcript is expressed in an adult human thymus cDNA library (Figure 3.2.5). To date, Northern blot analysis has shown UCP 3 mRNA to be present in skeletal muscle and BAT, although a wide array of tissues such as thymus, spleen, brain, heart, liver, intestine, lung, kidney, testis, uterus and
white adipose tissue has been analyzed (Gong et al., 1997 and Ricquier & Bouillaud, 2000). Our discovery of the existence of UCP 3 mRNA in human thymus contrasts with the results of Gong et al. (1997) who did not detect UCP 3 transcript in human thymus, using Northern blot analysis. This lack of detection of UCP 3 in human thymus, as reported by Gong et al. (1997), might indicate that UCP 3 mRNA is present in low abundance (less than several μg's) in the human thymus (as was the case for UCP 1 mRNA in rat thymus) and thus cannot be detected by Northern blotting. The novel finding that UCP 3 is expressed in human thymus is the first study to show that UCP 3 is associated with tissues/cells of the immune system.

The thymus is a primary site for production, development, growth and differentiation of lymphocytes. The majority of cells in the thymus are lymphocytes. With the discovery that UCP 1 is expressed in rat thymus and UCP 3 in human thymus, this study went on to determine whether the spleen, which derives its lymphocytes from the thymus, also expresses UCP’s. Our results show that UCP 1 was not detected in an adult human spleen cDNA library (Figure 3.2.6). This lack of UCP 1 mRNA detection in human spleen concurs favorably with other results obtained for mouse and rat spleen. Northern blot analysis did not detect UCP 1 mRNA in mouse (Ricquier & Bouillaud, 2000) and rat (Nègre-Salvayre et al., 1997) spleen. UCP 1’s expression in human spleen has not been previously investigated. Taken together, these results may reflect that UCP 1 is not expressed in mouse, rat or human spleen. However, as previously stated, UCP 1 is only associated with newborns and not adult humans (Boss et al., 1997). Therefore, one might not expect to see UCP 1 mRNA using an adult human spleen cDNA library. However, as other researchers show a lack of UCP 1 mRNA in mouse (Ricquier & Bouillaud, 2000) and rat (Nègre-Salvayre et al., 1997) spleen, it is probably logical to predict that UCP 1 transcript is not present in human spleen.

This study also shows that UCP 2 transcript is expressed in a human spleen cDNA library, using PCR methodology (Figure 3.2.6). The detection of UCP 2 transcript in human spleen confirms other findings in the literature. Northern blot analysis has previously shown that UCP 2 mRNA is present in mouse (Fluery et al.,
1997; Richard et al., 1999; Ricquier & Bouillaud, 2000; Pecqueur et al., 2001; Horvath et al., 2003), rat (Nègre-Salvayre et al., 1997; Jekabsons et al., 2001) and human (Gong et al., 1997) spleen. Due to the fact that UCP 2 has previously been detected in human spleen and rat and human thymus, using Northern blot analysis, it is probably safe to infer that UCP 2 mRNA is expressed in much higher abundance in these tissues compared to the parallel mRNA expression of UCP 1 in rat thymus and UCP 3 in human thymus and spleen.

Our results show the novel finding that UCP 3 mRNA is expressed in a human spleen cDNA library, using PCR methodology (Figure 3.2.6). The novel finding of UCP 3 mRNA in human spleen attained in this study is contradictory to previous findings in the literature. Several research groups have failed to detect UCP 3 mRNA in mouse (Richard et al., 1999; Ricquier & Bouillaud, 2000), rat (Jekabsons et al., 2001) or human (Gong et al., 1997) spleen, using northern blot analysis. The lack of detection of UCP 3 mRNA in spleen, as shown by these authors, may again infer that UCP 3 is present in low abundance (less than several μg's) and thus cannot be detected by Northern blot analysis due to its insensitivity at detecting low abundance mRNA's. By amplifying the human spleen cDNA library using PCR, this study maximized the chance of detecting UCP 3 transcript, even if present in low abundance. Our study is therefore the first study to show the expression of UCP 3 mRNA in other tissues apart from skeletal muscle and BAT. UCP 3 mRNA expression in human spleen, as well as human thymus, has substantiated UCP 3's possible involvement with tissues/cells of the immune system and therefore may imply a physiological role for UCP 3 associated with immune cell metabolism and function.

[3.4] Conclusion

We have shown for the first time the novel findings of UCP 1 mRNA in rat thymus, UCP 3 mRNA in both human thymus and spleen cDNA libraries using RT-PCR/PCR analysis. This study has therefore, for the first time, shown that UCP 1 and UCP 3 are associated with cells and tissues of the immune system and thus is the first study to imply a physiological role for UCP 1 and UCP 3 in immune cell
function. Our results also show that UCP 1 mRNA is not detected in an adult human thymus or spleen.

Our results have confirmed the presence of UCP 1, UCP 2 and UCP 3 mRNA in rat BAT. We subsequently show the lack of detection of UCP 1, UCP 2 and UCP 3 transcripts in rat liver. This study has also confirmed the presence of UCP 2 mRNA in rat and human thymus and human spleen.

However, for mRNA expression studies to have any functional significance in determining UCP 1, UCP 2 and UCP 3's physiological function(s), it will be necessary to investigate whether this mRNA expression manifests itself into the functional entity *i.e.* the protein. With this in mind, it is vital to complement our UCP 1, UCP 2 and UCP 3 mRNA expression studies with protein detection studies, so as to gain more insight into the functional relevance of these novel mRNA discoveries.
Chapter 4:

Detection of UCP 1 protein expression

using Western Blot analysis:
Chapter 4
Detection of UCP 1 protein expression using Western Blot analysis

[4.1] Introduction

Brown adipose tissue (BAT) is the major site of nonshivering thermogenesis (Heaton et al., 1978). Nonshivering thermogenesis is induced by cold, suggesting an important role for this tissue in adaptive energy balance (Klaus et al., 1991). UCP 1 is the key molecule involved in nonshivering thermogenesis in BAT. The thermogenic role of UCP 1 is due to its capacity to dissipate the proton electrochemical gradient generated during mitochondrial respiration producing heat instead of ATP. The development of high thermogenic capacity in BAT mitochondria isolated from cold-acclimated rats is characterized by a marked increase in the expression of UCP 1 protein (Desautels et al., 1978; Heaton et al., 1978; Swick & Swick, 1986; Sundin et al., 1987; Klaus et al., 1991; Nicholls & Rial, 1999; Nedergaard et al., 2001 & 2001a).

Up until this study, UCP 1 has only ever been associated with BAT mitochondria (Nicholls & Locke, 1984). Nibbelink et al. (2001) reported that UCP 1 was expressed in uterine longitudinal smooth muscle cells but this observation has been convincingly discredited by Rousset et al. (2003). In this study, we have already shown that UCP 1 mRNA is expressed in rat thymus (Figure 3.2.4, chapter 3) thus, we sought to investigate whether UCP 1 mRNA manifests itself into UCP 1 protein in the thymus.

We also sought to investigate whether UCP 1 protein is expressed in thymus cells (thymocyte) mitochondria. Starvation has previously been shown to decrease UCP 1 protein expression in BAT mitochondria (Rothwell et al., 1984; Trayhurn et al., 1988; Boss et al., 1998; Nedergaard et al., 2001). Therefore, we wanted to investigate the effects of starvation (48-hour) on UCP 1 protein expression in thymus and thymocyte mitochondria.

Throughout this chapter, BAT mitochondria isolated from rats kept at room temperature and rats acclimated to the cold for 28 days are used as positive controls for UCP 1 protein expression. Liver mitochondria and BAT mitochondria isolated
from UCP 1 knockout (KO) mice are used as negative controls for UCP 1 protein expression.

To quantify and observe changes in UCP 1 protein expression, we used western blot analysis. Commercial (Calbiochem) peptide antibodies reported to be sensitive to UCP 1 were tested for their sensitivity to UCP 1 and selectivity over UCP 2 and UCP 3 and other mitochondrial transporters.
Results

Figure 4.2.1 (A) shows a commercial anti-UCP 1 peptide antibody (Calbiochem) detecting UCP 1 in BAT mitochondria isolated from C57BL/6J wild-type (WT) mice (50-100-150μg). No UCP 1 protein was detected in BAT mitochondria isolated from C57BL/6J UCP 1 knockout (KO) mice or mouse liver mitochondria.

Figure 4.2.2 (A) shows the anti-UCP 1 peptide antibody (Calbiochem) detecting UCP 1 in BAT mitochondria isolated from rats kept at room temperature. No UCP 1 protein was detected in rat liver mitochondria or in inclusion bodies from E.coli expressing UCP 2. Figure 4.2.2 (B) shows a commercial anti-UCP 2 peptide antibody (Calbiochem) detecting UCP 2 protein in BAT mitochondria isolated from rats kept at room temperature. UCP 2 protein is also detected in inclusion bodies from E.coli expressing UCP 2. No UCP 2 protein was detected in rat liver mitochondria.

Figure 4.2.3 (A) shows the anti-UCP 1 peptide antibody (Calbiochem) detecting UCP 1 in BAT mitochondria isolated from C57BL/6J wild-type (WT) mice. No UCP 1 was detected in C57BL/6J UCP 1 knockout (KO) mice or mouse liver mitochondria. Figure 4.2.3 (B) shows our anti-UCP 3 peptide antibody detecting UCP 3 in BAT mitochondria isolated from C57BL/6J WT and UCP 1 KO mice. No UCP 3 protein was detected in mouse liver mitochondria. Figure 4.2.3 (C) shows that any changes in UCP 1 or UCP 3 expression in BAT mitochondria isolated from C57BL/6J WT, UCP 1 KO or mouse liver mitochondria cannot be accounted for by differences in lane loading, as indicated by an antibody to the β-subunit of the F₁-ATP synthase (F₁β).

Figure 4.2.4 (A) shows the anti-UCP 1 peptide antibody (Calbiochem) not detecting UCP 1 protein in yeast mitochondria expressing UCP 3, in gastrocnemius muscle mitochondria isolated from C57BL/6J wild-type (WT) or UCP 3 knockout (KO) mice and mouse liver mitochondria. Figure 4.2.4 (B) shows our anti-UCP 3
Figure 4.2.1: Anti-UCP 1 peptide antibody detects UCP 1 in BAT mitochondria from C57BL/6J wild-type (WT) but not from UCP 1 knockout (KO) mice.

BAT mitochondria from C57BL/6J wild-type and C57BL/6J UCP1 knockout mice were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of anti-UCP 1 (Calbiochem) peptide antibody (A). (See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa denotes the presence of UCP 1.)
Figure 4.2.2. Immunoblots showing the discriminatory nature of the anti-UCP 1 antibody (Calbiochem) over UCP 2.

BAT and liver mitochondria was isolated from fed rats as described in Section 2.8. Following isolation, mitochondria and inclusion bodies from E.coli expressing UCP 2 were subjected to SDS-PAGE (12% resolving gel) and western blotted with an anti-UCP 1 (Calbiochem) peptide antibody (A) and anti-UCP 2 (Calbiochem) peptide antibody (B). All antibodies were used at a 1:1,000 dilution. (See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa band denotes the presence of UCP 1 and UCP 2, respectively.)
Figure 4.2.3. Immunoblots showing the discriminatory nature of the anti-UCP 1 antibody (Calbiochem) over UCP 3 using BAT mitochondria isolated from C57BL/6J wild-type mice (WT) and C57BL/6J UCP 1 knockout (KO) mice.

Varying concentrations of BAT mitochondria from C57BL/6J wild-type (WT) mice and C57BL/6J UCP 1 knockout (KO) mice were subjected to SDS-PAGE (12% resolving gel) and western blotted with an anti-UCP 1 (Calbiochem) peptide antibody (A), an anti-UCP 3 antibody (B) and an antibody to the β-subunit of the F\textsubscript{1}-ATPase (F\textsubscript{1} β) (C). All antibodies were used at a 1:1,000 dilution. (See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~45 kDa band denotes the presence of UCP 1, UCP 3 and F\textsubscript{1} β respectively.
Figure 4.2.4. Immunoblots showing the discriminatory nature of the anti-UCP 1 antibody (Calbiochem) over UCP 3 using gastrocnemius muscle mitochondria from C57BL/6J wild-type (WT) and UCP 3 knockout (KO) mice.

Gastrocnemius muscle mitochondria from C57BL/6J wild-type (WT) and from C57BL/6J UCP 3 knockout (KO) 6-week old female mice were subjected to SDS-PAGE (12% resolving gel) and western blotted with an anti-UCP 1 (Calbiochem) peptide antibody (A), an affinity purified anti-UCP 3 peptide antibody (B) and an antibody to the β-subunit of the F₁-ATPase (F₁β) (C). All antibodies were used at a 1:1,000 dilution. (See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~45 kDa band denotes the presence of UCP 1, UCP 3 and F₁β respectively.)
peptide antibody detecting UCP 3 in yeast mitochondria expressing UCP 3 and in gastrocnemius muscle mitochondria isolated from C57BL/6J WT mice. No UCP 3 protein was detected in gastrocnemius muscle mitochondria isolated from C57BL/6J UCP 3 KO mice or mouse liver mitochondria. Figure 4.2.4 (C) shows the lack of detection of UCP 1 protein in BAT mitochondria isolated from UCP 1 KO mice, gastrocnemius muscle mitochondria isolated from C57BL/6J WT and UCP 3 KO mice and mouse liver mitochondria, cannot be accounted for by differences in lane loading as indicated by the F1β peptide antibody.

Figure 4.2.5 (A) shows the anti-UCP 1 peptide antibody (Calbiochem) detecting UCP 1 in BAT mitochondria isolated from rats acclimated to the cold for 28 days and from rats kept at room temperature. UCP 1 protein is also detected in thymocyte and thymus mitochondria isolated from fed and fasted rats. No UCP 1 protein was detected in liver mitochondria isolated from rats kept at room temperature. Figure 4.2.5 (B) shows that any changes in UCP 1 expression due to cold-acclimation or starvation, in BAT, thymocyte and thymus mitochondria cannot be accounted for by differences in lane loading, as indicated by an antibody to the β-subunit of the F1-ATP synthase. Figure 4.2.5 (C) shows the relative abundance of UCP 1 protein expression as a ratio to F1β, as determined by densitometry, for 3 separate experiments. Data is expressed as mean ± S.E.M. Cold-acclimation induced a significant 2-fold increase (P=0.005*, n=3) in UCP 1 protein expression in BAT mitochondria isolated from rats acclimated to the cold for 28 days, compared to rats kept at room temperature. Starvation induced no significant (NS) differences in UCP 1 expression in thymocyte or thymus mitochondria isolated from fasted rats, compared to fed controls. Statistical analysis was determined using an unpaired Students t-test. The bar chart also indicates the lack of expression of UCP 1 in rat liver mitochondria.

Figure 4.2.6 (A) shows our anti-UCP 1 peptide antibody (Eurogentec) detecting UCP 1 in BAT mitochondria isolated from C57BL/6J wild-type (WT) mice (50μg-150μg). No UCP 1 protein was detected in BAT mitochondria isolated from C57BL/6J UCP 1 knockout (KO) mice or mouse liver mitochondria.
Figure 4.2.5: UCP 1 is expressed in mitochondria isolated from BAT (4-week cold), BAT, Thymocyte (fed and fasted), Thymus (fed and fasted) and Liver.

Mitochondria was isolated from fed and fasted rats as described in Section 2.8. Following isolation, mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted an anti-UCP 1 (Calbiochem) peptide antibody (A) and an antibody to the β-subunit of the F₁ATPase (F₁β) (B). All antibodies were used at a 1:1,000 dilution. (See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~45 kDa band denotes the presence of UCP 1 and F₁β respectively. The bar chart (C) shows the relative abundance of UCP 1 protein expression as a ratio to F₁β, as determined by densitometry, for 3 separate preparations. Data is expressed as mean ± S.E.M. A significant 2-fold decrease (P=0.005*; n=3) in UCP 1 protein expression was observed in BAT mitochondria isolated from rats acclimated to the cold, when compared to BAT mitochondria isolated from rats kept at room temperature. Starvation induced no significant changes in UCP 1 protein expression in thymocyte or thymus mitochondria.
Figure 4.2.6: Our UCP 1 peptide antibody (Eurogentec) detects UCP 1 in BAT mitochondria from C57BL/6J wild-type (WT) but not UCP 1 knockout (KO) mice.

BAT mitochondria from C57BL/6J wild-type and UCP1 knockout mice were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of an anti-UCP 1 (Eurogentec) peptide antibody (A). (See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa denotes the presence of UCP 1.)
Figure 4.2.7 (A) shows our anti-UCP 1 peptide antibody (Eurogentec) detecting UCP 1 in BAT mitochondria isolated from rats acclimated to the cold for 28 days and from rats kept at room temperature. UCP 1 protein was also detected in thymocyte and thymus mitochondria isolated from fasted rats. No UCP 1 protein was detected in rat liver mitochondria. Figure 4.2.7 (B) shows that any changes in UCP 1 expression in BAT, thymocyte, thymus or liver mitochondria caused by cold-acclimation or fasting is independent of changes in mitochondrial loading, as indicated by F,β antibody.
Figure 4.2.7: UCP 1 expression in thymocyte and thymus mitochondria can be detected by our anti-UCP 1 peptide antibody (Eurogentec).

BAT, thymocyte and thymus mitochondria was isolated from fed rats as described in Section 2.8. Following isolation, mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted an anti-UCP 1 (Eurogentec) peptide antibody (A) and an antibody to the β-subunit of the F₁-ATPase (F₁β) (B). All antibodies were used at a 1:1,000 dilution. (See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~45 kDa band denotes the presence of UCP 1 and F₁β respectively.)
[4.3] Discussion

To observe and quantify changes in UCP 1 protein expression, discriminatory and sensitive antibodies are essential. In this study, we firstly sought to demonstrate that the commercial UCP 1 peptide antibody (Calbiochem) employed in this study was specific and selective for UCP 1 over UCP 2, UCP 3 and other mitochondrial transporters. This study shows the UCP 1 antibody (Calbiochem) detecting increasing amounts of UCP 1 in BAT mitochondria isolated from C57BL/6J wild-type (WT) but not from C57BL/6J UCP 1 knockout (KO) mice or mouse liver mitochondria (Figure 4.2.1). Our study has shown that UCP 2 protein is expressed in BAT mitochondria isolated from rats kept at room temperature (Figure 4.2.2). It has previously been shown that in BAT mitochondria isolated from C57BL/6J UCP 1 KO mice, UCP 2 mRNA levels were 5-14-fold higher than in the wild-type mice (Enerbäck et al., 1997; Matthias et al., 1999). Additionally, our study has also shown that UCP 3 protein is expressed in BAT mitochondria isolated from both C57BL/6J WT and UCP 1 KO mice (Figure 4.2.3). Despite the fact that UCP 2 and UCP 3 are expressed in BAT mitochondria isolated from UCP 1 KO mice, the Calbiochem UCP 1 antibody did not detect any UCP in these UCP 1 KO mice, thereby indicating its specificity for UCP 1 over UCP 2 and UCP 3. The lack of UCP 1 detection in liver mitochondria is to be expected as liver mitochondria do not constitutively express UCP 1 and was therefore used as a negative control tissue for the purpose of this study (Ricquier & Bouillaud, 1986 & 2000; Peachey et al., 1988; Matthias et al., 1999; Nedergaard et al., 2001). Taken together our results show that the Calbiochem UCP 1 peptide antibody is specific for UCP 1 over UCP 2, UCP 3 and other mitochondrial transporters in BAT mitochondria.

To further confirm the specificity of the Calbiochem UCP 1 peptide antibody, our results show that the Calbiochem UCP 1 antibody does not detect inclusion bodies from E.coli expressing UCP 2 (Figure 4.2.2). We go onto show a Calbiochem anti-UCP 2 peptide antibody can detect UCP 2 in BAT mitochondria and in inclusion bodies from E.coli expressing UCP 2. Therefore, we have confirmed the presence of UCP 2 in these inclusion bodies and show that the Calbiochem anti-UCP 1 peptide
antibody does not cross react with UCP 2. Furthermore, this study goes onto show that the Calbiochem UCP 1 antibody does not detect protein in yeast expressing UCP 3, nor does it detect protein in UCP 3-containing gastrocnemius muscle mitochondria isolated from C57BL/6J WT and UCP 3 KO mice. The latter result is to be expected as UCP 1 is not expressed in isolated rat skeletal muscle mitochondria (Ricquier & Bouillaud, 1986 & 2000). Taken together, these results show that the Calbiochem UCP 1 peptide antibody is sensitive and specific for UCP 1 over UCP 2, UCP 3 and all other members of the mitochondrial anion transport family.

With this specific Calbiochem UCP 1 antibody in hand, we were able to observe the expected increase in UCP 1 protein expression in BAT mitochondria upon cold-acclimation (Figure 4.2.5). Our results show a significant 2-fold increase (P=0.005) in UCP 1 protein expression in BAT mitochondria isolated from cold-acclimated rats, when compared to BAT mitochondria isolated from rats kept at room temperature. Our finding correlates well with results obtained by other authors in the literature (Desautels et al., 1978; Heaton et al., 1978; Swick & Swick, 1986; Sundin et al., 1987; Klaus et al, 1991; Nicholls & Rial, 1999; Nedergaard et al., 2001).

In chapter 3, we show the novel finding that UCP 1 mRNA is present in rat thymus (Figure 3.2.4). For this result to have any functional significance, we sought to investigate whether UCP 1 mRNA was translated into the functional entity i.e. the protein. Our results show that UCP 1 protein is constitutively expressed in thymus mitochondria isolated from both fed and fasted rats. We also detected UCP 1 protein in mitochondria isolated from thymus cells i.e. thymocytes. Detection of UCP 1 protein in thymocyte and thymus mitochondria was achieved using both the Calbiochem and Eurogentec UCP 1 peptide antibodies (Figure 4.2.5 & 4.2.7). Under conditions of acute starvation (48-hour), we could not detect any significant (P<1.0) difference in the abundance of UCP 1 protein in thymocyte or thymus mitochondria. It is interesting to note that fasting is associated with an increased metabolic efficiency and a decrease in UCP 1 protein expression and activity in BAT mitochondria (Rothwell et al., 1984; Trayhurn et al., 1988; Boss et al., 1998;
Nedergaard et al., 2001). However, the fact that starvation induced no significant change in UCP 1 protein expression in thymus mitochondria may imply that UCP 1 plays a different role in thymus compared to its role in BAT. Taken together, this is the first study to show that UCP 1 is associated with cells/tissues of the immune system.

[4.4] Conclusion

In conclusion, our study has shown the novel finding that UCP 1 protein is expressed in thymocyte and thymus mitochondria. Starvation (48-hour) did not induce a change in UCP 1 expression in either thymocyte or thymus mitochondria. The exact function or role of UCP 1 in thymus mitochondria has yet to be resolved. No matter what, the presence of UCP 1 protein in thymus mitochondria has exposed a novel route into the bioenergetics and metabolic function of these immune cells.
Chapter 5:

Detection of UCP 3 protein expression
using Western Blot analysis
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Detection of UCP 3 protein expression
using Western Blot analysis

[5.1] Introduction

The function of UCP 3 is still contentious but insights into its physiological function can be obtained from observing its pattern of expression. Northern blot, RT-PCR and PCR analysis has been key in identifying possible locations and functions of UCP 3 mRNA (see Chapter 3; Boss et al., 1997 & 1998; Vidal-Puig et al., 1997; Cadenas et al., 1999; Giacobino et al., 1999; Pedraza et al., 2001; Cabrero et al., 1999, 2000 & 2001). Detection of UCP 3 transcript is useful but it does not necessarily represent the expression of the functional entity i.e. the protein. Several lines of evidence support the fact that UCP mRNA levels do not correlate with protein expression levels. UCP 2 transcript studies has shown that UCP 2 mRNA is expressed in brain, heart, skeletal muscle, stomach, lung, spleen, kidney, testis, uterus, white adipose tissue (WAT), BAT, thymus, placenta, kidney, pancreas, prostate, testis, ovary and the colon (Boss et al., 1997; Gong et al., 1997; Ricquier & Bouillaud, 2000). However protein expression studies show UCP 2 is confined to namely spleen, lung, stomach, kidney and thymus (Ricquier et al., 2000; Pecqueur et al., 2001; Krauss et al., 2002; Echtay et al., 2001a). Similarly, Pecqueur et al. (2001) shows that UCP 2 protein expression in stomach mitochondria is <10 times the amount of UCP 2 protein expressed in spleen, even though the relative amounts of UCP 2 mRNA in both organs are similar. Pecqueur et al. (2001) has also demonstrated that the increase in UCP 2 protein in lung and stomach upon fasting or lipopolysaccharide (LPS) treatment is not accompanied by an increase of UCP 2 mRNA. Cadenas et al. (1999) showed that starvation increased UCP 2 and UCP 3 mRNA levels more than 5-fold and 4-fold, respectively, whereas UCP 3 protein levels increased by only 2-fold in rat skeletal muscle mitochondria. Sivitz et al. (1999) and Giacobino et al. (2001) showed that UCP 3 mRNA levels increased 4-fold upon starvation in gastrocnemius muscle, with protein levels increasing only 2-fold in gastrocnemius muscle mitochondria in experiments carried out in parallel.
As one of the aims of this study was to try and elucidate UCP 3's physiological function, we needed to quantify the functional entity i.e. the protein. To quantify and observe changes in UCP 3 protein expression, sensitive and discriminatory antibodies are essential. To date, very few publications have focused on UCP 3 protein expression, despite the fact that commercial antibodies are available from Calbiochem, Chemicon International, Lilly and Alpha-Diagnostics. The main reason for this is probably due to the absence of reliable and specific immunological tools (i.e. antibodies) that are selective and discriminatory at detecting UCP 3 in vivo. Consequently, UCP 3 peptide antibodies have been developed in-house by other research groups (Vidal-Puig et al., 2000; Hesselink et al., 2003). However, the sensitive and discriminatory nature of these commercial and in-house UCP 3 antibodies to other UCP's and to other mitochondrial transporters have yet to be adequately demonstrated.

With all this in mind, we designed a UCP 3 peptide, generated by Eurogentec, against which an antibody was raised in rabbit (see section 2.17, chapter 2). Research performed in our laboratory has shown this UCP 3 peptide antibody to be sensitive and discriminatory for UCP 3 over UCP 1, UCP 2 and other mitochondrial transporters (Cunningham et al., 2003). Our research group has shown our UCP 3 peptide antibody detecting mouse, rat and human forms of UCP 3 expressed in E.coli, COS cells, yeast expression systems, isolated mitochondria and whole muscle groups (Cunningham et al., 2003). In addition, we also showed that our UCP 3 antibody will only detect UCP 3 that is coincident with the mitochondrial fraction of rat skeletal muscle homogenates and not peroxisomal, nuclear or cytosolic and microsomal fractions (Cunningham et al., 2003).

Therefore, having established the discriminatory nature of our polyclonal UCP 3 peptide antibody, we sought to investigate UCP 3 protein expression in isolated mitochondria under various physiological conditions.

Cumulative data has shown that UCP 3 transcript and protein is expressed in skeletal muscle (Boss et al., 1997; Ježek et al., 1999; Langin et al., 1999; Clapham et al., 2000; Gong et al., 2000; Li et al., 2000; Ricquier & Bouillaud, 2000; Vidal-Puig et al., 2000; Zhou et al., 2000; Cadenas et al., 2002; Harper et al., 2002; Cunningham et al., 2003; Hesselink et al., 2003) and BAT (Boss et al., 1997; Ježek
et al., 1999; Langin et al., 1999; Clapham et al., 2000; Gong et al., 2000; Li et al., 2000; Ricquier & Bouillaud, 2000; Nedergaard et al., 2001; Harper et al., 2002; Cunningham et al., 2003). Liver is known not to constitutively express UCP 3 transcript (Boss et al., 1997; Gong et al., 1997; Emilsson et al., 1998; Hodorý et al., 1998; Richard et al., 1999) or protein (Ježek et al., 1999; Gong et al., 2000; Simonyan et al., 2001; Echtay et al., 2002). Therefore, for the purpose of this study, skeletal muscle and BAT mitochondria were used as positive control tissues and liver as a negative control tissue for our UCP 3 peptide antibody.

Starvation has been shown to increase UCP 3 mRNA (Boss et al., 1998; Weigle et al., 1998; Brun et al., 1999; Cadenas et al., 1999; Sivitz et al., 1999; Samec et al., 1999; Moreno et al., 2003) and protein (Cadenas et al., 1999; Sivitz et al., 1999; Moreno et al., 2003) levels in mouse and rat skeletal muscle. Short-term cold exposure has also been shown to increase UCP 3 transcript (Lin et al., 1998; Samec et al., 2000; Schrauwen et al., 2002; Wang et al., 2003) and protein (Simonyan et al., 2001; Wang et al., 2003) in rat skeletal muscle. Furthermore, administration of thyroid hormone to mice and rats has been shown to increase UCP 3 mRNA (Jekabsons et al., 1999; Lanni et al., 1999; Reitman et al., 1999) and protein (Lombardi et al., 2002) in skeletal muscle.

With a selective and discriminatory UCP 3 peptide antibody at hand, we sought to confirm the effects of fasting, cold-exposure and thyroid hormone on UCP 3 protein expression in isolated rat skeletal muscle mitochondria. In addition, we sought to investigate whether the novel findings of UCP 3 transcript in human thymus and human spleen manifest itself into UCP 3 protein. We also wanted to investigate whether UCP 3 protein was expressed in thymus cells (thymocytes) and spleen cells (reticulocytes, monocytes and lymphocytes). Furthermore, we wanted to determine whether starvation had any effect on UCP 3 protein expression in these novel UCP 3-expressing tissues.

Finally, lymphoid atrophy is a well-recognized consequence of nutritional deprivation in animals, including humans (Howard et al., 1999). It has long been known that the size of the spleen and thymus varies according to the state of nutrition of the body, being large in fed and small in starved animals. This study sought to
investigate this lymphoid atrophy by comparing the weights of a spleen and thymus from fasted rats, compared to fed controls.
[5.2] Results

UCP 3 protein expression using Western blot analysis

The in vivo site of UCP 3 protein is skeletal muscle mitochondria. Figure 5.2.1 (A) shows our anti-UCP 3 peptide antibody detecting UCP 3 in yeast mitochondria expressing UCP 3 and in gastrocnemius muscle mitochondria isolated from C57BL/6J wild-type (WT) mice. No UCP 3 protein was detected in gastrocnemius muscle mitochondria isolated from C57BL/6J UCP 3 knockout (KO) mice or mouse liver mitochondria. Figure 5.2.1 (B) shows that the specificity of our anti-UCP 3 peptide antibody in detecting UCP 3 in C57BL/6J WT but not in C57BL/6J UCP 3 KO mice, cannot be accounted for by differences in lane loading as indicated by an antibody to the β-subunit of the F₁-ATP synthase (F₁β).

The most abundant UCP in BAT is UCP 1, representing 10% of the mitochondrial inner membrane protein of BAT mitochondria from cold-acclimated hamsters and rats. Therefore to ensure our anti-UCP 3 peptide antibody was not detecting native UCP 1, BAT mitochondria isolated from C57BL/6J wild-type (WT) and UCP 1 knockout (KO) mice were probed with our UCP 3 antibody. Figure 5.2.2 (A) shows our anti-UCP 3 peptide antibody detecting UCP 3 in yeast mitochondria expressing UCP 3, in BAT mitochondria isolated from C57BL/6J wild-type (WT) and C57BL/6J UCP 1 knockout (KO) mice. UCP 3 was not detected in mouse liver mitochondria. Figure 5.2.2 (B) indicates that changes in UCP 3 expression in BAT mitochondria isolated from C57BL/6J WT and UCP 1 KO mice and mouse liver mitochondria, cannot be accounted for by differences in lane loading as indicated by F₁-β antibody. Figure 5.2.2 (C) shows the relative abundance of UCP 3 as a ratio to F₁β in C57BL/6J WT, UCP 1 KO and liver mouse mitochondrial samples, as determined by densitometry. Data is expressed as mean ± S.E.M for at least 3 preparations. No significant (NS) difference (P<1.0) in UCP 3 expression was observed in BAT mitochondria isolated from either C57BL/6J WT or UCP 1 KO mice, as determined by an unpaired Students t-test. No UCP 3 protein is expressed in mouse liver mitochondria isolated from fed mice.
Figure 5.2.1 Anti-UCP 3 peptide antibody detects UCP 3 in isolated gastrocnemius mitochondria from wild-type (WT) but not from UCP 3 knockout (KO) mice.

Gastrocnemius muscle mitochondria from C57BL/6J wild-type (WT) and from C57BL/6J UCP 3 knockout (KO) 6-week old female mice were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of an antibody to the β-subunit of the F₁-ATPase (F₁β)(B). See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~45 kDa band denotes the presence of UCP 3 and F₁β respectively.
Figure 5.2.2: Anti-UCP 3 peptide antibody detects equal amounts of UCP 3 in isolated BAT mitochondria from C57BL/6J wild-type (WT) and UCP 1 knockout (KO) mice.

BAT mitochondria from C57BL/6J wild-type and C57BL/6J UCP1 knockout mice were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of an antibody to the β-subunit of the F$_1$-ATPase (F$_1$β) (B). See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~45 kDa band denotes the presence of UCP 3 and F$_1$β respectively. The bar chart (C) shows the relative abundance of UCP 3 protein expression as a ratio to F$_1$β, as determined by densitometry, for 3 separate preparations. Data is expressed as mean ± S.E.M. No significant difference in UCP 3 expression was observed between WT and UCP 1 KO mice (P<1.0). Absence of error bars indicated the errors were smaller than the size of the symbol.
Figure 5.2.3 (A) shows our affinity purified antibody detecting UCP 3 in yeast mitochondria expressing UCP 3. No UCP 3 protein was detected in liver mitochondria isolated from fed rats or inclusion bodies from *E.coli* transformed to express UCP 2. Figure 5.2.3 (B) shows a commercial anti-UCP 2 peptide antibody (Calbiochem) detecting UCP 2 expressed in inclusion bodies expressed in *E.coli*. No UCP 2 protein was detected in yeast mitochondria expressing UCP 3 or liver mitochondria isolated from fed rats. Figure 5.2.3 (C) shows that the lack of UCP 2 and UCP 3 protein expression in liver mitochondria isolated from fed rats, cannot be accounted for by the absence of mitochondria as indicated by the presence of F\textsubscript{1}-\beta antibody.

Figure 5.2.4 (A) shows our affinity purified anti-UCP 3 peptide antibody detecting UCP 3 in yeast mitochondria expressing UCP 3, in skeletal muscle mitochondria isolated from fed and fasted rats and in BAT mitochondria isolated from rats acclimated to the cold for 28 days. No UCP 3 protein was detected in liver, heart, whole brain, kidney or lung mitochondria. Figure 5.2.4 (B) shows that the lack of expression of UCP 3 in liver, heart, whole brain, kidney and lung mitochondria is not a result of insufficient amounts of mitochondria loaded, as indicated by an antibody to cytochrome oxidase subunit III.

Figure 5.2.5 shows that UCP 3 protein expression in isolated skeletal muscle mitochondria is starvation-sensitive. Figure 5.2.5 (A) shows our anti-UCP 3 peptide antibody detecting UCP 3 in yeast mitochondria expressing UCP 3. No UCP 3 protein was detected in liver mitochondria isolated from fed rats. Our anti-UCP 3 peptide antibody detected UCP 3 in skeletal muscle mitochondria isolated from fed and fasted rats. Figure 5.2.5 (B) shows that differences in UCP 3 expression in skeletal muscle mitochondria isolated from fed and fasted rats, cannot be accounted for by differences in lane loading, as indicated by COIII antibody. Figure 5.2.5 (C) shows the relative abundance of UCP 3 protein expression as a ratio to COIII in skeletal muscle and liver mitochondrial samples, as determined by densitometry. Data is expressed as mean ± S.E.M for at least 4 preparations. The results show a significant 2.8-fold increase in UCP 3 expression (P=0.001;* n=4) in skeletal muscle.
Yeast mitochondria expressing UCP 3, rat liver mitochondria and inclusion bodies from *E. coli* transformed to express UCP 2 were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A), a 1:1,000 dilution of anti-UCP 2 peptide antibody (B) and a 1:1,000 dilution of an antibody to the β-subunit of the F$_1$-ATPase (F$_1$β) (C). (See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~ 45 kDa band denotes the presence of UCP 3, UCP 2 and F$_1$β respectively.)
Figure 5.2.4: Anti-UCP 3 peptide antibody detects UCP 3 in skeletal muscle mitochondria isolated from fed and fasted rats and in BAT mitochondria isolated from rats acclimated to the cold for 4 weeks.

Mitochondria were isolated as described in section 2.8. Following isolation, mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of anti-COIII peptide antibody (B). (See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~25 kDa band denotes the presence of UCP 3 and COIII respectively.)
Skeletal muscle and liver mitochondria were isolated as described in section 2.8 from fed and fasted rats. Following isolation, mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of anti-COIII peptide antibody (B). See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~25 kDa band denotes the presence of UCP 3 and COIII respectively. The bar chart (C) shows the relative abundance of UCP 3 protein expression as a ratio to COIII, as determined by densitometry, for 5 separate preparations. Data is expressed as mean ± S.E.M. The significance with respect to UCP 3's 2.8-fold increased expression in skeletal muscle mitochondria between fed and fasted states, calculated by an unpaired Students t-test, was P=0.001* (n=5).
mitochondria isolated from fasted rats, when compared to fed controls. Statistical analysis was determined using an unpaired Students t-test. The bar chart also indicates the lack of expression of UCP 3 in rat liver mitochondria isolated from fed rats. Similarly, no UCP 3 protein has been detected in liver mitochondria isolated from fasted rats (results not shown).

Figure 5.2.6 shows that UCP 3 expression in rat skeletal muscle mitochondria is sensitive to changes in temperature acclimation over a period of 8 weeks. Figure 5.2.6 (A) shows our anti-UCP 3 peptide antibody detecting UCP 3 in yeast mitochondria expressing UCP 3 and in rat skeletal muscle mitochondria isolated from rats housed at 20°C ± 2°C, 30°C ± 2°C and 8°C ± 2°C over a period of 8 weeks. Our anti-UCP 3 peptide antibody did not detect UCP 3 in rat liver mitochondria isolated from rats housed at 20°C. Figure 5.2.6 (B) shows that changes in UCP 3 expression resulting from changes in temperature acclimation, is totally independent of the amount of mitochondria loaded, as indicated by COIII antibody. Anti-COII peptide antibody corrected for differences in UCP 3 intensity as a result of variations in mitochondrial lane loading. Figure 5.2.6 (C) shows the relative abundance of UCP 3 expression as a ratio to COIII, as determined by densitometry, for 3 separate preparations. Data is expressed as mean ± S.E.M. UCP 3 expression significantly decreases 1.4-fold (P=0.0047*, n=3) in skeletal muscle mitochondria isolated from rats acclimated at 30°C for 8 weeks, compared to skeletal muscle mitochondria from rats acclimated to 20°C for 8 weeks. There is a significant 2.3-fold increase (P=0.0023**, n=3) in UCP 3 expression in skeletal muscle mitochondria isolated from rats acclimated at 8°C for 8 weeks, compared to skeletal muscle mitochondria isolated from rats acclimated at 30°C for 8 weeks. Finally, there is a significant 1.6-fold increase (P=0.0113***, n=3) in UCP 3 expression in skeletal muscle mitochondria isolated from rats acclimated at 8°C, compared to skeletal muscle mitochondria from rats acclimated at 20°C for 8 weeks. Statistical analysis was determined using an unpaired Students t-test. The bar chart also indicates the lack of UCP 3 expression in liver mitochondria isolated from rats housed at 20°C.
Figure 5.2.6: Effects of temperature changes on UCP 3 expression in isolated skeletal muscle mitochondria.

Skeletal muscle mitochondria were isolated from rats acclimated to $20^\circ C \pm 2^\circ C$, $30^\circ C \pm 2^\circ C$ and $8^\circ C \pm 2^\circ C$ for 8 weeks. Following isolation, mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of anti-COIII peptide antibody (B). See sections 2.13 & 2.14 for a detailed description of these procedures. A $\sim 33$ kDa and $\sim 25$ kDa band denotes the presence of UCP 3 and COIII respectively. The bar chart (C) shows the relative abundance of UCP 3 protein expression as a ratio to COIII, as determined by densitometry, for 3 separate preparations. Data is expressed as mean ± S.E.M. The significance with respect to UCP 3’s increased expression in skeletal muscle mitochondria between different temperature states, calculated by an unpaired Students t-test, was $P=0.0047$ (n=3)*, $P=0.0023$ (n=3)**, $P=0.0113$ (n=3)***. 
Figure 5.2.7 shows that UCP 3 expression increases in skeletal muscle mitochondria isolated from hyperthyroid rats, compared to euthyroid controls. Figure 5.2.7 (A) shows our anti-UCP 3 peptide antibody detecting UCP 3 in yeast mitochondria expressing UCP 3. UCP 3 protein was not detected in liver mitochondria isolated from euthyroid rats. Our anti-UCP 3 peptide antibody detected UCP 3 in skeletal muscle mitochondria isolated from euthyroid rats, fasted rats (48-hour starvation) and from hyperthyroid rats. Figure 5.2.7 (B) shows that any differences in UCP 3 expression in skeletal muscle mitochondria isolated from euthyroid, fasted and hyperthyroid rats, cannot be accounted for by differences in lane loading, as indicated by COIII. Figure 5.2.7 (C) shows the relative abundance of UCP 3 protein expression as a ratio to COIII, as determined by densitometry, for 4 separate preparations. Data is expressed as mean ± S.E.M. Our results show a significant 2.3-fold increase (P=0.002**, n=4) in UCP 3 expression in skeletal muscle mitochondria isolated from hyperthyroid rats, when compared to euthyroid controls. Statistical analysis was determined using an unpaired Students t-test. The bar chart also shows that no UCP 3 protein is present in liver mitochondria isolated from euthyroid rats.

Figure 5.2.8 shows that UCP 3 protein is expressed in thymus mitochondria, with its expression being starvation sensitive. Figure 5.2.8 (A) shows our anti-UCP 3 peptide antibody detecting UCP 3 in yeast mitochondria expressing UCP 3 and in thymus mitochondria from fed and fasted rats. No UCP 3 was detected in liver mitochondria isolated from fed rats. Figure 5.2.8 (B) shows that any changes in UCP 3 expression in thymus mitochondria isolated from fed and fasted rats are independent of changes in mitochondrial loading, as indicated by F1β antibody. Figure 5.2.8 (C) shows the relative abundance of UCP 3 protein expression in thymus mitochondria as a ratio to F1β, as determined by densitometry, for 3 separate preparations. Data is expressed as mean ± S.E.M. Our results show a significant 1.5-fold increase in UCP 3 expression (P=0.03*, n=3) in thymus mitochondria from fasted rats compared to fed controls. Statistical analysis was determined using an unpaired Students t-test.
Figure 5.2.7: UCP 3 expression increases in skeletal muscle mitochondria isolated hyperthyroid rats, when compared to euthyroid controls.

Skeletal muscle mitochondria were isolated from euthyroid, 48-hr starved and hyperthyroid rats as described in section 2.8. Following isolation, mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of anti-COIII peptide antibody (B). See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~25 kDa band denotes the presence of UCP 3 and COIII respectively. The bar chart (C) shows the relative abundance of UCP 3 protein expression as a ratio to COIII, as determined by densitometry, for 4 separate preparations. Data is expressed as mean ± S.E.M. The significance with respect to UCP 3’s increased expression in skeletal muscle mitochondria between euthyroid and hyperthyroid states, calculated by an unpaired Students t-test, was P<0.002 (n=4)*.
Figure 5.2.8: 48-hour starvation induces a 1.5-fold increase in UCP 3 expression in isolated thymus mitochondria.

Thymus mitochondria were isolated from fed and fasted rats as described in Section 2.8. Following isolation, thymus mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of an antibody to the β-subunit of the F₁-ATPase (F₁β) (B). See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~45 kDa band denotes the presence of UCP 3 and F₁β respectively. The bar chart (C) shows the relative abundance of UCP 3 protein expression as a ratio to F₁β, as determined by densitometry, for 3 separate preparations. Data is expressed as mean ± S.E.M. The significance with respect to UCP 3’s increased expression in thymus mitochondria between fed and fasted states, calculated by an unpaired Students t-test, was P=0.03 (n=3)*
Figure 5.2.9 shows that thymocyte mitochondria also express a starvation sensitive UCP 3 protein. Figure 5.2.9 (A) shows our anti-UCP 3 peptide antibody detecting UCP 3 protein in yeast mitochondria expressing UCP 3 and in thymocyte mitochondria isolated from fed and fasted rats. No UCP 3 protein was detected in liver mitochondria isolated from fed rats. Figure 5.2.9 (B) shows that any changes in UCP 3 expression in thymocyte mitochondria between fed and fasted states, is independent of changes in mitochondrial loading, as indicated by F1β antibody. Figure 5.2.9 (C) shows the relative abundance of UCP 3 protein expression in thymocyte mitochondria as a ratio to F1β, as determined by densitometry, for 3 separate preparations. Data is expressed as mean ± S.E.M. The significance with respect to UCP 3’s 3-fold increased expression in thymocyte mitochondria, upon starvation, as calculated by an unpaired Students t-test, was P=0.001*(n=3).

Figure 5.2.10 shows that UCP 3 protein is expressed in spleen mitochondria and that its expression starvation insensitive. Figure 5.2.10 (A) shows that our anti-UCP 3 peptide antibody detects UCP 3 in yeast mitochondria expressing UCP 3 and in spleen mitochondria isolated from fed and fasted rats. No UCP 3 protein was detected in liver mitochondria isolated from fed rats. Figure 5.2.10 (B) shows that changes in UCP 3 expression in spleen mitochondria isolated from fed and fasted rats cannot be accounted for by differences in lane loading, as indicated by F1β antibody. Figure 5.2.10 (C) shows the relative abundance of UCP 3 protein expression as a ratio to F1β, as determined by densitometry, for 3 separate experiments. Data is expressed as mean ± S.E.M. No significant differences (NS) were observed in UCP 3 protein expression in spleen mitochondria isolated from fed and fasted rats (P<1.0). Statistical analysis was determined using an unpaired Students t-test.

Having established that UCP 3 protein is expressed in spleen mitochondria, we sought to investigate whether UCP 3 is associated with the major cell types of the spleen, namely reticulocytes, monocytes and lymphocytes. We also wanted to investigate whether starvation would affect UCP 3 expression in these cell types. Figure 5.2.11 shows that UCP 3 protein is expressed in these spleen cells but that
Figure 5.2.9: 48-hour starvation induces a 3-fold increase in UCP 3 expression in isolated thymocyte mitochondria.

Thymocyte mitochondria were isolated from fed and fasted rats as described in Section 2.9. Following isolation, thymocyte mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of an antibody to the β-subunit of the F₁-ATPase (F₁ β) (B). See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~45 kDa band denotes the presence of UCP 3 and F₁ β respectively. The bar chart (C) shows the relative abundance of UCP 3 protein expression as a ratio to F₁ β, as determined by densitometry, for 3 separate preparations. Data is expressed as mean ± S.E.M. The significance with respect to UCP 3’s increased expression in thymocyte mitochondria between fed and fasted states, calculated by an unpaired Students t-test, was P=0.001 (n=3)*
Figure 5.2.10: 48-hour starvation does not increase UCP 3 expression in isolated spleen mitochondria.

Spleen mitochondria were isolated from fed and fasted rats as described in Section 2.8. Following isolation, spleen mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of an antibody to the β-subunit of the $F_1$-ATPase ($F_1β$) (B). See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~45 kDa band denotes the presence of UCP 3 and $F_1β$ respectively. The bar chart (C) shows the relative abundance of UCP 3 protein expression as a ratio to $F_1β$, as determined by densitometry, for 3 separate preparations. Data is expressed as mean ± S.E.M. No significant differences in UCP 3 expression was observed in spleen mitochondria between fed and fasted states ($P$=0.3; $n$=3).
Figure 5.2.11: UCP 3 is homogenously expressed in all cell types of the spleen isolated from fed or fasted rats.

Spleen cell mitochondria were isolated from fed and fasted rats as described in Section 2.10. Following isolation, mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of affinity purified anti-UCP 3 peptide antibody (A) and a 1:1,000 dilution of an antibody to the F1-ATPase (B). See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa and ~45 kDa band denotes the presence of UCP 3 and F1 β respectively. The bar chart (C) shows the relative abundance of UCP 3 protein expression as a ratio to F1 β, as determined by densitometry, for 3 separate preparations. Data is expressed as mean ± S.E.M. No significant differences in UCP 3 expression was observed in homogenate, reticulocytes or monocyte mitochondrial fractions between fed or fasted states. However, the significance with respect to UCP 3's increased expression in lymphocyte mitochondria between fed and fasted states, calculated by an unpaired Students t-test, was P=0.0021 (n=3)*
starvation only induces an increase in UCP 3 expression in spleen lymphocyte mitochondria. Figure 5.2.11 (A) shows our anti-UCP 3 peptide antibody detecting UCP 3 in yeast mitochondria expressing UCP 3. Our anti-UCP 3 peptide antibody also detects UCP 3 in spleen cell homogenate, reticulocyte, monocyte and lymphocyte mitochondrial fractions, isolated from fed and fasted rats. No UCP 3 protein was detected in liver mitochondria isolated from fed rats. Figure 5.2.11 (B) shows that any differences in UCP 3 expression in homogenate, reticulocyte, monocyte or lymphocyte mitochondria, upon starvation, is independent of changes in mitochondrial loading, as indicated by F$_{1}$$\beta$ antibody. Figure 5.2.11 (C) shows the relative abundance of UCP 3 as a ratio to F$_{1}$$\beta$, as determined by densitometry, for at least 3 separate experiments. Data is expressed mean ± S.E.M. Starvation (48-hours) does not significantly (NS) augment UCP 3 expression in spleen cell homogenate, reticulocyte or monocyte mitochondria, whereas UCP 3 expression increases significantly (P=0.0021*, n=3) in spleen lymphocyte mitochondria isolated from fasted rats, compared to fed controls. Statistical analysis was determined using an unpaired Students t-test. The bar chart also indicates the lack of UCP 3 expression in liver mitochondria isolated from fed rats.

Figure 5.2.12 shows the commercial anti-calbiochem UCP 3 peptide antibody detecting UCP 3 in mitochondria isolated from skeletal muscle, spleen, thymocyte and thymus of fed rats.

Table 5.2 summarizes the effects of starvation, temperature acclimation and hyperthyroidism on UCP 3 protein expression. Starvation induces a significant increase in UCP 3 protein expression in mitochondria isolated from skeletal muscle, thymus, thymocyte and spleen lymphocytes. Starvation caused no increase in UCP 3 expression in spleen, spleen homogenate, spleen reticulocyte and spleen monocyte mitochondria. UCP 3 protein was detected in BAT mitochondria isolated from rats acclimated to the cold for 4 weeks. UCP 3 expression was significantly increased in skeletal muscle mitochondria of rats after long-term cold exposure (8°C). UCP 3 expression was decreased in skeletal muscle mitochondria isolated from rats.
Figure 5.2.12: UCP 3 expression in mitochondria isolated from spleen, thymocyte and thymus using a commercial anti-UCP 3 peptide antibody.

Mitochondria were isolated from fed rats as described in Section 2.8. Following isolation, mitochondria were subjected to SDS-PAGE (12% resolving gel) and western blotted with a 1:1,000 dilution of anti-UCP 3 calbiochem antibody overnight at 4°C. See sections 2.13 & 2.14 for a detailed description of these procedures. A ~33 kDa band denotes the presence of UCP 3.
Table 5.2: A summary of UCP 3’s protein expression patterns under different physiological conditions.

Table 5.2 (A) shows the effects of starvation (48-hour) on UCP 3 protein expression in a variety of tissues. Table 5.2 (B) shows the effects of temperature acclimation on UCP 3 protein expression in BAT, skeletal muscle and liver mitochondria. Table 5.2 (C) shows the effects of euthyroid and hyperthyroidism on UCP 3 protein expression in skeletal muscle and liver mitochondria.

**Symbols:** “++”: detection of UCP 3 protein; “+”: decrease in UCP 3 protein expression relative to ++; “+++”: increase in UCP 3 protein expression relative to ++; “-”: No UCP 3 protein was detected; “ND”: UCP 3 protein expression was not detected.

### Table 5.2 (A)

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<th>Fasted State</th>
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</tr>
<tr>
<td>Skeletal Muscle</td>
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<td>+++</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thymus</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Thymocyte</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Spleen</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Spleen Homogenate</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
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<td>+</td>
</tr>
<tr>
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<td>+</td>
</tr>
<tr>
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### Table 5.2 (B)

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<td>++</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
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<td>++</td>
<td>+++</td>
</tr>
<tr>
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<td>-</td>
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</tr>
</tbody>
</table>

### Table 5.2 (C)

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<tr>
<th>Source</th>
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<tbody>
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</tr>
<tr>
<td>Liver</td>
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</tr>
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</table>
acclimated to 30°C for 8 weeks. Our results also show that UCP 3 expression is increased in skeletal muscle mitochondria isolated from hyperthyroid rats.

Figure 5.2.13 (A) shows that starvation (48-hour) results in a significant 1.2-fold decrease in the weight of the spleen, from 0.53g ± 0.016 (n=3) in fed rats to 0.45g ± 0.003 (n=3) in fasted rats (P=0.008*; n=3). Figure 5.2.13 (B) shows that starvation (48-hour) results in a significant 1.4-fold decrease in the weight of the thymus, from 0.31g ± 0.01 (n=3) in fed rats to 0.22g ± 0.006 (n=3) in fasted rats (P=0.0015**; n=3). All data is expressed as mean ± S.E.M, for at least 3 separate preparations.
Figure 5.2.13: Spleen and Thymus decrease in weight (grams) after 48-hr starvation.

Spleen and thymus were carefully excised from the rat and weighed from fed and fasted rats as described in section 2.8. (A) shows the weight of a spleen decreases 1.17-fold upon starvation from 0.53g ± 0.016 (n=3) in fed rats to 0.45g ± 0.003 (n=3) in 48-hr starved rats (P=0.008*; n=3). (B) shows the weight of a thymus decreases 1.4-fold upon starvation from 0.31g ± 0.01 (n=3) in fed rats to 0.22g ± 0.006 (n=3) in 48-hr starved rats (P=0.0015**; n=3). Absence of error bars indicates errors were smaller than the size of the symbol.
[5.3] Discussion

In this study, we further demonstrate that our UCP 3 peptide antibody is specific and selective for UCP 3 over UCP 1, UCP 2 and other mitochondrial transporters, results we have now published in Cunningham et al. (2003). This study shows our polyclonal UCP 3 peptide antibody detecting UCP 3 in gastrocnemius mitochondria of C57BL/6J wild-type but not C57BL/6J UCP 3 knock-out mice (Figure 5.2.1). We also show our UCP 3 peptide antibody detecting UCP 3 to the same extent in BAT mitochondria isolated from C57BL/6J wild-type and C57BL/6J UCP 1 knockout mice (Figure 5.2.2). As UCP 1 is the most abundant UCP in BAT, our results show that our UCP 3 antibody does not detect native UCP 1 but is selective for UCP 3 over UCP 1. Furthermore our results show that our UCP 3 peptide does not detect inclusion bodies expressing UCP 2 (Figure 5.2.3). Taken together, these results show that our UCP 3 peptide antibody is sensitive for UCP 3 over UCP 1, UCP 2 and all other members of the mitochondrial anion transport family.

In the study presented here, our UCP 3 antibody has detected and thus confirmed the expression of UCP 3 protein in rat skeletal muscle and BAT mitochondria (Figure 5.2.4) and lack of UCP 3 in liver. This study also confirms that starvation augments UCP 3 protein expression in skeletal muscle. Therefore, as our data for skeletal muscle and BAT mitochondria are consistent with those in the literature, we used both tissues act as clear positive controls for our UCP 3 antibody. Similarly, liver mitochondria acts as a negative control tissue for our UCP 3 antibody. Since the discovery of UCP 3, its mRNA and protein expression has only been associated with skeletal muscle and BAT, despite investigating a wide variety of tissues such as brain, heart, liver, intestine, lung, spleen, thymus, kidney, testis, uterus, white adipose tissue, prostrate, ovary and colon (Boss et al., 1997; Gong et al., 1997; Vidal-Puig et al., 1997; Ricquier & Bouillaud, 2000). Several research groups have shown that UCP 3 transcript is expressed in mouse and rat skeletal muscle (Boss et al., 1997; Langin et al., 1999; Clapham et al., 2000; Ricquier & Bouillaud, 2000; Vidal-Puig et al., 2000) and BAT (Boss et al., 1997; Langin et al., 1999; Clapham et al., 2000; Ricquier & Bouillaud, 2000; Nedergaard et al., 2001). Similarly, many authors have shown that UCP 3 protein is expressed in mouse and
rat skeletal muscle (Ježek et al., 1999; Gong et al., 2000; Li et al., 2000; Zhou et al., 2000; Vidal-Puig et al., 2000; Cadenas et al., 2002; Harper et al., 2002; Cunningham et al., 2003; Hesselink et al., 2003) and BAT mitochondria (Ježek et al., 1999; Gong et al., 2000; Li et al., 2000; Harper et al., 2002; Cunningham et al., 2003). Our study also shows that our UCP 3 antibody did not detect UCP 3 protein in mitochondria isolated from brain, kidney and lung. However, these tissues are known to contain UCP 2 transcript (Boss et al., 1997; Gong et al., 1997; Ricquier & Bouillaud, 2000) and protein (Echtay et al., 2001; Pecqueur et al., 2001). Therefore, this lack of detection of UCP 3 in brain, kidney and lung mitochondria further emphasizes the specificity of our UCP 3 antibody over UCP 2 and other members of the mitochondrial anion transporter family.

As mentioned earlier, we confirmed that starvation increases UCP 3 protein expression in isolated rat skeletal muscle mitochondria (Figure 5.2.5), but we went on further and quantified this increase in UCP 3 expression (2.8-fold increase after 48-hour starvation). Our findings concur favorably with those in the literature. Several other research groups have shown that starvation increases UCP 3 mRNA expression in skeletal muscle from mouse (Boss et al., 1998; Brun et al., 1999) and rats (Wiegle et al., 1998; Cadenas et al., 1999; Samec et al., 1999; Moreno et al., 2003). Similarly, other research groups have shown that starvation increased UCP 3 protein expression levels ~2-fold in rat skeletal muscle and gastrocnemius muscle mitochondria (Cadenas et al., 1999; Sivitz et al., 1999; Moreno et al., 2003). The increase in UCP 3 protein expression during starvation, at a time when energy expenditure is decreased, does not support a primary role for UCP 3 in mouse skeletal muscle thermogenesis. Weigle et al. (1998) has shown that an elevation in free fatty-acid levels in rats, stimulate UCP 3 mRNA expression in skeletal muscle. As 48-hour starvation and free fatty-acids increase UCP 3 mRNA and protein expression, one may hypothesise that fatty-acids are positive modulators of UCP 3. These findings are more consistent with a role for UCP 3 in the regulation of fatty-acid metabolism than in nonshivering thermogenesis and energy balance (see general discussion chapter).

Results obtained in this study also show that UCP 3 protein expression is temperature sensitive. This study shows UCP 3 expression increased 1.6-fold in
skeletal muscle mitochondria isolated from rats acclimated to the cold for 8-weeks (8°C), compared to control rats kept at room temperature (20°C) (Figure 5.2.6). We also show that UCP 3 expression decreases 1.4-fold in skeletal muscle mitochondria kept at warm temperatures (30°C) for 8 weeks when compared to rats kept at room temperature (20°C). We also show there is a 2.3-fold difference in UCP 3 in skeletal muscle mitochondria isolated cold-acclimated rats when compared to warm adapted rats (30°C). These results compliment results of several other authors in the literature. Other research groups have previously shown that short-term cold-exposure (24-hour) increased the expression of UCP 3 transcript in rat skeletal muscle (Lin et al., 1998; Wang et al., 2003). Short-term cold-exposure (24-hour) has been also been shown to increase UCP 3 protein levels in rat skeletal muscle mitochondria (Simonyan et al., 2001; Wang et al., 2003). Despite the fact that our results compliment those of Simonyan et al. (2001) and Wang et al. (2003), our results contrast with those of Boss et al. (1998) who showed that UCP 3 mRNA levels in mouse skeletal muscle did not increase with short-term cold exposure (24-hour). To date, no other research group has investigated the long-term effects of cold-exposure on UCP 3 protein expression. Similarly, no other research group has investigated the effects of warm (30°C) acclimation on UCP 3 expression. Interestingly, cold exposure like starvation, has been shown to correlate nicely with increasing free fatty-acids (Simonyan et al., 2001). As both factors increase UCP 3 protein expression, one may again postulate a role for UCP 3 in lipid metabolism rather than in thermogenesis (see general discussion).

The role of the thyroid gland in the regulation of metabolic rate has been known since the last century (Reitman et al., 1999). Thyroid hormone increases energy expenditure, in part by lowering metabolic efficiency and is therefore a major determinant of basal metabolism. We predict that if UCP 3 is involved in regulating metabolic flux then UCP 3 expression might be sensitive to thyroid status. This study shows that hyperthyroidism induces a 2.3-fold increase in UCP 3 protein expression in skeletal muscle mitochondria, when compared to euthyroid controls. Our results confirm those of several research groups in the literature. Several authors have shown that administration of thyroid hormone increased UCP 3 mRNA expression 4-6-fold above euthyroid levels in skeletal muscle of mice and rats (Gong et al., 1997;
Jekabsons et al., 1999; Lanni et al., 1999; Reitman et al., 1999). Lombardi et al. (2002) showed that UCP 3 protein expression increased 3.5 fold in skeletal muscle mitochondria isolated from hyperthyroid rats, compared to euthyroid controls. These authors also showed that the hypothyroidism-hyperthyroidism transition is accompanied by increases in the endogenous levels of mitochondrial free fatty-acids, all of which correlate with an increase in UCP 3 protein expression. Consequently, to date, this study has shown significant increases in UCP 3 protein expression associated with starvation, cold-acclimation and thyroid treatment. Such physiological conditions are all associated with an increase in the levels of endogenous free fatty-acids. Therefore, taken together, we speculate that UCP 3 is involved in fatty-acid metabolism rather than in thermogenesis but this latter speculation will be discussed in more detail in the general discussion chapter.

To date, UCP 2 has been the only UCP to be associated with immune-tissues (Gong et al., 1997; Nègre-Salvayre et al., 1997; Krauss et al., 2002). Krauss et al. (2002) has shown that UCP 2 mediates a proton leak in intact thymocytes. In our endeavors to further investigate this proton leak, we determined what other UCP’s, if any, were also associated with the thymus. We have previously shown the novel finding that UCP 3 mRNA is expressed in human thymus (Figure 3.2.5, chapter 3) and we wanted to investigate further whether this UCP 3 mRNA was translated into protein. This study shows the novel finding that UCP 3 protein is constitutively expressed in thymus and thymocyte mitochondria (Figure 5.2.8 and 5.2.9). Furthermore, starvation (48-hour) induces a 1.5- fold and 3-fold increase in UCP 3 protein expression in thymus and thymocyte mitochondria, respectively, which correlates nicely with a possible role for UCP 3 in fatty-acid metabolism in thymus and thymocyte. To date, no other research group have investigated the presence of UCP 3 protein in thymus mitochondria.

The thymus is a lymphocyte rich tissue, which plays a central role in the creation of a fully functional immune system. Its major function is to provide the appropriate milieu within which cells of the T-lymphocyte lineage can develop, proliferate and mature. With the discovery that UCP 3 transcript and protein is expressed in human and rat thymus respectively, we wanted to determine whether
the spleen, which derives its lymphocytes from the thymus, also expresses UCP 3. The spleen is the largest lymphatic organ, with its cellular components mainly consisting of lymphocytes, macrophages and monocytes (MacPherson, 1973). The spleen is also known to contain a small number of mitochondrial-containing reticulocytes (Takano-Ohmuro et al., 2000). This study has previously shown the novel finding that UCP 3 transcript is expressed in human spleen and we wanted to see whether UCP 3 mRNA manifests itself into protein. Our results show the novel finding that UCP 3 protein is constitutively expressed in spleen mitochondria (Figure 5.2.10). Starvation had no effect on UCP 3 expression in spleen mitochondria which may imply that UCP 3’s role in spleen is different to its role in skeletal muscle and thymus mitochondria. To date, no other research group have investigated the expression of UCP 3 protein in spleen. Therefore, the expression of UCP 3 protein in rat spleen mitochondria compliments our UCP 3 transcript result showing that UCP 3 mRNA was expressed in human spleen. The association of UCP 3 with yet another tissue of the immune system may imply a role for UCP 3 in immune cell metabolism and function.

However, cellular fractionation of rat spleen shows that UCP 3 protein is associated with mitochondria from the major cell types present, namely reticulocytes, monocytes and lymphocytes fractions (Figure 5.2.11). Starvation has no effect on UCP 3 protein expression in whole spleen cell homogenate, reticulocyte or monocyte fractions. However, starvation increased UCP 3 protein expression in spleen lymphocytes. This is the first study to show that UCP 3 is localized preferentially in the lymphocytes of the spleen, with its expression being starvation-sensitive.

As our UCP 3 peptide antibody is unique to our research laboratory, we sought to determine whether any commercially available anti-UCP 3 peptide antibody would confirm our novel findings. Interestingly, the calbiochem anti-UCP 3 peptide antibody detected UCP 3 protein in skeletal muscle, spleen, thymocyte and thymus mitochondria (Figure 5.2.12), albeit to lower expression levels than what our UCP 3 antibody detected. Nevertheless, it is surprising that no other research group has discovered the presence of UCP 3 protein in thymus, thymocyte and spleen mitochondria, seeing that these commercial antibodies are readily available.
Lymphoid atrophy results in the shrinkage of lymphoid tissues caused by nutritional deprivation to an animal or human (Howard et al., 1999). It has long been known that the size of the spleen varies according to the nutritional state of the body, being large in highly fed and small in starved animals. However the disproportionate loss of lymphoid tissue with starvation is more pronounced in the thymus than the spleen (Howard et al., 1999). This study sought to confirm this lymphoid atrophy by determining the weights of both spleen and thymus in fasted rats, compared to fed controls. Our results show that the weight of a spleen and thymus decreases 1.2-fold and 1.4-fold, respectively, upon starvation (Figure 5.2.13). This finding is consistent with lymphoid atrophy being more pronounced in the thymus than the spleen (Howard et al., 1999). The marked reduction in thymic size has been associated with the loss of thymic lymphocytes (Ritter & Crispe, 1992). The increase in UCP 3 expression on starvation in lymphocyte-rich tissues i.e. thymus and thymocyte mitochondria and spleen lymphocyte mitochondria, may suggest a role for UCP 3 in starvation, regulation of lymphoid atrophy or osmotic changes associated with starvation.

[5.4] Conclusion

This study has shown the novel findings that UCP 3 protein is expressed in thymus and spleen mitochondria. Our results also show the novel findings that UCP 3 protein is expressed in thymus cells (thymocytes) and spleen cells (reticulocytes, monocytes and lymphocytes). This study went onto show that starvation increased UCP 3 expression in thymus, thymocyte and spleen lymphocyte mitochondria. Starvation had no effect on UCP 3 expressed in spleen, spleen cell reticulocyte and monocyte mitochondria.

This study has also confirmed that presence of UCP 3 protein in skeletal muscle and BAT mitochondria and confirms that liver mitochondria contains no UCP 3 protein. Furthermore, this study has confirmed the effects of starvation, cold-exposure and thyroid treatment on UCP 3 expression in skeletal muscle.
mitochondria. This study also shows that starvation induced lymphoid atrophy in spleen and thymus.

The role of UCP 3 in immune function will now be a matter of investigation. The increase in UCP 3 expression on starvation in thymus, thymocyte and spleen lymphocyte mitochondria is consistent with UCP’s increased expression in skeletal muscle mitochondria. Taken together, these results may imply a role for UCP 3 in fatty-acid oxidation or thymic atrophy. The fact that UCP 3 is associated with the primary and secondary lymphocyte producing tissues, the thymus and spleen respectively, may postulate a role for UCP 3 in the developing T-lymphocyte. All of these roles are purely speculative and will require further investigation. No matter what, our novel findings have uncovered a novel bioenergetic route for further investigation into immune cell metabolism and function that’s associated with UCP 3.
Chapter 6

Application of a radiolabeled purine nucleotide binding assay to UCP containing mitochondria.
Chapter 6
Application of a radiolabeled purine nucleotide binding assay to UCP containing mitochondria.

[6.1] Introduction
The unique mechanisms underlying the enormous thermogenic capacity of brown adipose tissue (BAT) have been investigated since the 1960's. The first step towards identification of the uncoupling component of BAT, found that BAT mitochondria were highly permeable to protons and halides and that purine nucleotides decreased both proton and halide conductance (Nicholls, 2001). The discovery that purine nucleotides abrogated both proton and halide transport in BAT mitochondria, led researchers to determine the mechanism in which they do so. The binding site for the purine nucleotide was found by photo-affinity labeling of BAT mitochondrial membranes with radioactive $\alpha$-azido-ATP (Heaton et al., 1978). Heaton et al. (1978) found that the nucleotides bind to a high-affinity site on the cytosolic side of the mitochondrial inner membrane, which is independent of the adenine nucleotide translocator. Heaton et al. (1978) also showed that nucleotides recouple by binding to a 32 kDa protein on the mitochondrial inner membrane. The 32 kDa protein was identified as uncoupling protein (also known as thermogenin and now UCP 1) (Lin & Klingenberg, 1982). UCP 1 determines the thermogenic potential of BAT (Nicholls & Locke, 1984). UCP 1 is a GDP-binding protein and is as such the cause of the high GDP-binding capacity of BAT mitochondria. The specific binding of radiolabelled GDP has been used extensively as an indication of the thermogenic capacity of BAT (Sundin & Cannon, 1980; Nedergaard & Cannon, 1985; Sundin et al., 1987). Radiolabeled and fluorescent-labeled purine nucleotide binding assays are the most sensitive and specific methods for determining the presence of UCP 1 when compared to western blotting techniques (Milner et al., 1988).

With the discovery of a family of UCP's i.e. UCP 2 and UCP 3 (Fluery et al., 1997; Boss et al., 1997), this study sought to investigate whether mitochondria constitutively expressing UCP 2 and UCP 3 can bind purine nucleotides akin to UCP 1. There is some evidence in the literature to support the hypothesis that UCP 2 and
UCP 3 can bind purine nucleotides. Chemical modification and site-directed mutagenesis studies have shown that amino acid residues reported to be essential for the binding of GDP by UCP 1, are 75-80% conserved in UCP 2 and UCP 3 (long form only; UCP 3L) (Klaus et al., 1991; Klingenberg & Huang, 1999). Chemical modification studies have shown the contiguous sequence EGPAAFFKG, associated with the sixth putative helix, to be involved in purine nucleotide binding in mouse and rat UCP 1 (Klaus et al., 1991). Similar contiguous sequences EGPRAFYKG and EGPTAFYKG are found at equivalent positions in UCP 2 and UCP 3, thereby implying that UCP 2 and UCP 3 may bind purine nucleotides (Figure 1.10, chapter 1). Additionally, site-directed mutagenesis studies have shown that three arginines resembling Arg 83 (84) (Helix 2), Arg 182 (183) (Helix 4) and Arg 276 (277) (Helix 6) in the mouse (and rat) UCP 1 sequence are essential for purine nucleotide binding (Modriansky et al., 1997; Klingenberg & Huang, 1999; Porter, 2001). Equivalent arginines exist in equivalent positions in UCP 2 and UCP 3. Consequently, amino acid residues involved in purine nucleotide binding to UCP 1 are conserved in UCP 2 and UCP 3 thus suggesting these proteins bind purine nucleotides.

Most of the evidence to date showing that UCP 2 and UCP 3 can bind purine nucleotides have come from E.coli and heterologous yeast expression systems. Jekabsons et al. (2002) showed that recombinant human UCP 2 ectopically expressed and solubilised in bacterial (E.coli) inclusion bodies bind purine and pyrimidine nucleoside triphosphates with low micromolar affinity. Zackova et al. (2003) also showed that E.coli or yeast expressing UCP 2 and UCP 3 exhibit high affinity [3H] GTP binding, similar to UCP 1. However, data showing purine nucleotide binding to UCP 2 and UCP 3 containing mitochondria has been somewhat lacking.

In attempting to elucidate whether UCP 2 and UCP 3 can bind purine nucleotides, this study sought to firstly apply a radioligand-binding assay specific and sensitive for UCP 1 in BAT mitochondria and secondly, use this assay as a template for investigating specific [3H] GDP binding to UCP 2 and UCP 3 containing mitochondria. The [3H] GDP binding assay employed in this study was set up according to the method of Scarpace et al. (1991). One of the most important considerations in any radioligand-binding assay is the determination of specific
binding. In this study, specific binding can be defined as binding to UCP 1’s purine nucleotide binding site. Non-specific binding is any other observed binding. Operationally, nonspecific binding is the observed binding in the presence of an appropriate excess of unlabeled ligand (i.e. GDP) to block fully the receptors of interest. Nonspecific binding includes binding of the radioligand to glass fibre filters, absorption to the tissue and dissolution in the membrane lipids. Specific binding is calculated as the difference between the total and nonspecific binding. In this study, binding data was obtained using saturation binding analysis. In a saturation experiment, the amount of radioligand-receptor complex is measured as a function of the free radioligand concentration using an equation describing a rectangular hyperbola (see section 2.24). The parameters obtained from this type of experiment are the affinity, usually expressed as the dissociation constant $K_D$, and the maximal number of binding sites, $B_{MAX}$.

Similar radioligand-binding assays have been successfully employed to determine saturable binding to UCP 1 in BAT mitochondria (Sundin & Cannon, 1980; Nedergaard & Cannon, 1985; Gribskov et al., 1986; Swick & Swick, 1986; Sundin et al., 1987; Milner et al., 1988; Peachey et al., 1988; Scarpace et al., 1991; Echtay et al., 1998; Huang et al., 1998). Additionally, fluorescent nucleotide probes have been very successful in determining purine nucleotide binding to UCP 1 in BAT mitochondria, yeast and *E.coli* expression systems and to UCP 2 in *E.coli* expression systems (Huang & Klingenberg, 1995; Huang & Klingenberg, 1995a; Huang & Klingenberg, 1996; Echtay et al., 1997; Echtay et al., 1998; Jekabsons et al., 2002).

The aims of this chapter was to firstly use BAT mitochondria, isolated from rats acclimated to the cold for 28 days, a regime known to increase UCP 1’s thermogenic capacity and thus its GDP binding capabilities, as a positive control tissue. Liver mitochondria have been shown not to constitutively express UCP 1, UCP 2 or UCP 3, and therefore were used as a negative control tissue for the purpose of this study (Ricquier & Bouillaud, 2000).

Secondly, our study and previous studies have shown that UCP 3 transcript and protein are constitutively expressed in rat skeletal muscle and skeletal muscle mitochondria (Figure 5.2.4 & 5.2.5, chapter 5; Boss et al., 1997; Ježek et al., 1999;
Langin et al., 1999; Clapham et al., 2000; Gong et al., 2000; Li et al., 2000; Ricquier & Bouillaud, 2000; Vidal-Puig et al., 2000; Zhou et al., 2000; Cadenas et al., 2002; Harper et al., 2002; Cunningham et al., 2003; Hesselink et al., 2003). This study has unequivocally shown the novel finding that UCP 1 transcript and protein is constitutively expressed in thymus and thymus mitochondria (Figure 3.2.4; chapter 3 & Figure 4.2.5; chapter 4). Other studies have shown that UCP 2 transcript and protein is expressed in thymus mitochondria (Gong et al., 1997; Nègre-Salvayre et al., 1997; Krauss et al., 2002). This study has also found the novel finding that UCP 3 transcript and protein is constitutively expressed in thymus and thymus mitochondria (Figure 3.2.5; chapter 3 and Figure 5.2.8; chapter 5). Finally, spleen mitochondria are known to contain UCP 2 protein (Pecqueur et al., 2001; Echtay et al., 2002). Furthermore, the study presented here has shown the novel finding that UCP 3 transcript and protein is expressed in spleen and spleen mitochondria (Figure 3.2.6; chapter 3 & Figure 5.2.10; chapter 5). Therefore, we sought to investigate whether UCP 2 and UCP 3 containing mitochondria can bind purine nucleotides and the tissues we choose as a source of these mitochondria were skeletal muscle, thymus and spleen mitochondria. As UCP 1 is a GDP-binding protein in BAT mitochondria, we were also particularly interested in investigating whether UCP 1 is a GDP-binding protein in thymus mitochondria.
[6.2] Results

Figure 6.2.1 shows saturable $[^3]$H GDP binding to BAT mitochondria isolated from rats acclimated to the cold for 28 days. The data were fitted to an equation describing a rectangular hyperbola, using non-weighted, non-linear least square regression analysis. The data clearly show saturable binding up to 6 μM of $[^3]$H GDP. The $K_d$ and $B_{\text{MAX}}$ values obtained were $0.7 \pm 0.3$ μM (n=3) and $1640 \pm 204$ (n=3) pmoles bound/mg protein respectively. Non-specific, non-saturable binding was determined in the presence of unlabelled GDP (1.5mM).

In figure 6.2.2, the amount of specific GDP binding was calculated as the excess amount of $[^3]$H GDP found on the filter after correcting for trapped buffer by the use of $[^14]$C sucrose. $[^14]$C sucrose cannot penetrate the mitochondrial membrane and therefore functions as an extra-mitochondrial marker. Saturable $[^3]$H GDP binding is clearly observed in BAT mitochondria isolated from rats acclimated to the cold for 28 days after correcting for $[^14]$C sucrose (figure 6.2.2). The $K_d$ and $B_{\text{MAX}}$ values obtained were $0.7 \pm 0.3$ μM (n=3) and $1572 \pm 215$ (n=3) pmoles bound/mg protein respectively.

Table 6.1 shows a comparison of the binding parameters obtained with $[^3]$H GDP ($\pm [^14]$C sucrose) to fresh and freeze-thawed BAT mitochondria isolated from rats acclimated to the cold for 28 days. The $K_d$ and $B_{\text{MAX}}$ values obtained with $[^3]$H GDP and $[^3]$H GDP ($\pm [^14]$C sucrose) are very similar in the respective fresh and freeze-thawed BAT preparations. The $B_{\text{MAX}}$ values obtained using freeze-thawed BAT mitochondria (940 ± 128 pmoles bound/mg protein; n=5) are significantly lower (P=0.02*; n=5) than the $B_{\text{MAX}}$ value calculated using fresh BAT mitochondrial preparations (1640 ± 204 pmoles bound/mg protein; n=3). No significant difference was observed between the $K_d$ values for fresh and freeze-thawed BAT mitochondrial preparations (P<1.0), thus indicating that UCP’s affinity for GDP is conserved in both fresh and freeze-thawed states.
Figure 6.2.1: Saturable $[^3]$H GDP binding to BAT mitochondria isolated from rats acclimated to the cold for 28 days.

BAT mitochondria were isolated from rats acclimated to the cold for 28 days as described in section 2.8. BAT mitochondria (50 μg) isolated from cold-acclimated rats were incubated with $[^3]$H GDP (11.0 Ci/mmol; 0.1-6.0 μM) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5 mM) as described in section 2.22. Specific binding was calculated from the difference between total and non-specific binding. The values for specific binding represent the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate. Absence of error bars indicates the errors were smaller than the size of the symbol. $K_D$ and $B_{MAX}$ values were obtained by fitting the data to an equation describing a rectangular hyperbola using the computer program Sigma plot. The line represents best fit.

The $K_D$ and $B_{MAX}$ values obtained were $0.7 ± 0.3$ μM (n=3) and $1640 ± 204$ (n=3) pmoles bound/mg protein respectively.
Figure 6.2.2: Saturable $[^3]$H GDP binding, in the presence of $[^{14}]$C sucrose, to BAT mitochondria isolated from rats acclimated to the cold for 28 days.

BAT mitochondria were isolated from rats acclimated to the cold for 28 days as described in section 2.8. BAT mitochondria (50 μg) isolated from cold-acclimated rats was incubated with $[^3]$H GDP (11.0 Ci/mmol; 0.1-6.0 μM) and $[^{14}]$C sucrose (250 μCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5 mM) as described in section 2.22.

Specific binding was calculated from the difference between total and non-specific binding. The values for specific binding represent the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate. Absence of error bars indicates the errors were smaller then the size of the symbol. $K_D$ and $B_{MAX}$ values were obtained by fitting the data to an equation describing a rectangular hyperbola using the computer graphics program Sigma plot. The line represents best fit.

The $K_D$ and $B_{MAX}$ values obtained were 0.7 ± 0.3 μM (n=3) and 1572 ± 215 (n=3) pmoles bound/mg protein respectively.
### Table 6.1: Comparison of the $[^3\text{H}]$ GDP ($±[^1\text{C}]$ Sucrose) binding parameters to fresh and freeze-thawed BAT mitochondria isolated from rats acclimated to the cold for 28 days.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Fresh BAT mitochondria</th>
<th>Freeze-thawed BAT mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_D$ (µM)</td>
<td>$B_{MAX}$ (pmols bound/mg protein)</td>
</tr>
<tr>
<td>$[^3\text{H}]$ GDP</td>
<td>0.7 ± 0.3*</td>
<td>1640 ± 204$^d$</td>
</tr>
<tr>
<td>($±[^1\text{C}]$ Sucrose)</td>
<td>0.7 ± 0.3</td>
<td>1572 ± 215</td>
</tr>
</tbody>
</table>

Fresh and freeze-thawed BAT mitochondria (50 µg) were incubated with $[^3\text{H}]$ GDP (11.0 Ci/mmol; 0.1-6.0 µM) and/or $[^1\text{C}]$ Sucrose (250 µCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5mM) as described in Section 2.22. Specific binding was calculated from the difference between the total and non-specific binding. Data was fitted to an equation describing a rectangular hyperbola using the computer program Sigma Plot and the $K_D$ and $B_{MAX}$ binding parameters were determined. The above table summarises the values obtained for these parameters. Each value represents the mean ± S.E.M of at least three experiments, each experiment performed in triplicate. No significant difference in the $K_D$ and $B_{MAX}$ values were observed using the $[^3\text{H}]$ GDP and $[^3\text{H}]$ GDP (+ $[^1\text{C}]$-sucrose) binding assays. The significance with respect to fresh versus freeze-thawed $K_D$ and $B_{MAX}$ values, calculated by the Students $t$-test, was $P< 1.0^*$ and $P< 0.02^*$ where indicated.
GDP binding was also determined for liver mitochondria. As liver mitochondria is known not to express UCP's, it was used as a negative control tissue for the radiolabelled binding assays employed in this study. Figure 6.2.3 shows $[^3]H$ GDP binding to liver mitochondria to be non-saturable and thus, prevented the accurate determination of $B_{\text{MAX}}$ and $K_D$ values.

Skeletal muscle mitochondria have been shown to constitutively express UCP 3 protein. Starvation (48-hour) has also been shown to increase UCP 3 protein levels 2-3 fold. Circumstantial evidence from primary sequence studies indicates that UCP 2 and UCP 3 can bind purine nucleotides. With all this in mind, we sought to investigate $[^3]H$ GDP binding to UCP 3 containing skeletal muscle mitochondria isolated from fed and fasted rats. Figure 6.2.4 shows $[^3]H$ GDP binding to skeletal muscle mitochondria to be non-saturable. Consequently, determination of the binding parameters, $B_{\text{MAX}}$ and $K_D$, were unattainable.

As temperature is an important determinant in optimizing binding assay conditions, $[^3]H$ GDP binding to freeze-thawed BAT mitochondria, previously isolated from rats acclimated to the cold for 28 days, was measured at various temperatures (0°C, 10°C, 21°C and 37°C) (Table 6.2). $[^3]H$ GDP binding on ice (0°C) and 10°C revealed a decrease in the $K_D$ values respectively, compared to the $K_D$ values obtained at room temperature (21°C) and at 37°C. $K_D$ values for freeze-thawed BAT mitochondria on ice (0°C) and at 10°C were determined to be 9.8 ± 3 μM and 1.4 ± 0.6 μM respectively, compared to $K_D$ values of 0.9 ± 0.4 μM and 0.8 ± 0.3 μM for $[^3]H$ GDP binding at room temperature and 37°C respectively (mean ± S.E.M, of three independent determinations). The $B_{\text{MAX}}$ values (within error) remained unaffected by changes in temperature.

One of the most remarkable features of nucleotide binding to UCP 1 is its strong pH dependence (Klingenberg & Huang, 1999). To ensure pH was not a factor in the lack of $[^3]H$ GDP binding to UCP 3 containing mitochondria, this study sought to investigate $[^3]H$ GDP binding to skeletal muscle mitochondria by varying the pH of the binding assay medium (Table 6.3). Table 6.3 shows that the $K_D$ and $B_{\text{MAX}}$
Figure 6.2.3: Low-affinity $[^3]H$ GDP binding to isolated liver mitochondria.

Liver mitochondria were isolated from rats kept at room temperature, as described in section 2.8. Liver mitochondria (50 µg) was incubated with $[^3]H$ GDP (11.0Ci/mmol; 0.1-6.0 µM) and $[^14]C$ sucrose (250 µCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5 mM) as described in section 2.22. Specific binding was calculated from the difference between total and non-specific binding. Attempts to determine $K_D$ and $B_{MAX}$ values were achieved by fitting the data to an equation describing a rectangular hyperbola using the computer program Sigma plot. However, determination of the binding parameters $K_D$ and $B_{MAX}$ were unattainable, as the specific binding was not found to be saturable. Data is expressed as the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate.
Figure 6.2.4: Low-affinity $[^3]H$ GDP binding to Skeletal Muscle mitochondria isolated from fed and fasted rats.

Skeletal muscle mitochondria were isolated from fed and fasted rats, as described in section 2.8. Skeletal muscle mitochondria (50 µg) were incubated with $[^3]H$ GDP (11.0 Ci/mmol; 0.1-6.0 µM) and $[^14]C$ sucrose (250 µCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5 mM) as described in section 2.22. Specific binding was calculated from the difference between total and non-specific binding.

Attempts to determine $K_D$ and $B_{MAX}$ values were achieved by fitting the data to an equation describing a rectangular hyperbola using the computer program Sigma plot. However, determination of the binding parameters $K_D$ and $B_{MAX}$ were unattainable, as the specific binding was not found to be saturable. Data is expressed as the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate.
<table>
<thead>
<tr>
<th>Assay Temp</th>
<th>$K_D$ (µM)</th>
<th>$B_{MAX}$ (pmoles bound/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$0^\circ$C</td>
<td>9.8 ± 3</td>
<td>954 ± 176</td>
</tr>
<tr>
<td>$10^\circ$C</td>
<td>1.4 ± 0.6</td>
<td>816 ± 140</td>
</tr>
<tr>
<td>$21^\circ$C</td>
<td>0.9 ± 0.4</td>
<td>1009 ± 119</td>
</tr>
<tr>
<td>$37^\circ$C</td>
<td>0.8 ± 0.3</td>
<td>942 ± 128</td>
</tr>
</tbody>
</table>

Table 6.2: $[^3H]$ GDP binding to freeze-thawed BAT mitochondria, previously isolated from rats acclimated to the cold for 28 days, is temperature dependent.

Freeze-thawed BAT mitochondria (50 µg) were incubated with $[^3H]$ GDP (11.0 Ci/mmol; 0.1-6.0 µM) for 15 minutes at varying temperatures of $0^\circ$C, $10^\circ$C, $21^\circ$C and $37^\circ$C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5mM) as described in Section 2.22. Specific binding was calculated from the difference between the total and non-specific binding. Data was fitted to an equation describing a rectangular hyperbola using the computer program Sigma Plot and the $K_D$ and $B_{MAX}$ binding parameters were determined. The above table summarises the values obtained for these parameters. Each value represents the mean ± S.E.M of at least three experiments, each experiment performed in triplicate.
<table>
<thead>
<tr>
<th>pH</th>
<th>$K_D$ (μM)</th>
<th>$B_{MAX}$ (pmoles bound/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal Muscle mitochondria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>6.8</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>7.1</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>7.4</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 6.3: Non-saturable (NS) $[^3]H$ GDP binding to isolated skeletal muscle mitochondria over a range of pH’s.

Skeletal Muscle mitochondria (50 μg) were incubated with $[^3]H$ GDP (11.0 Ci/mmol; 0.1-6.0 μM) at various pH’s for 15 minutes at 37°C in the absence (Total binding) and presence (Non-specific binding) of unlabelled GDP (1.5 mM) as described in Section 2.22. Determination of the binding parameters $K_D$ and $B_{MAX}$ were unattainable at any given pH, as the specific binding was not found to be saturable (NS; non-saturable).
values could not be determined as the specific binding was not found to be saturable (NS; non-saturable) at any given pH (pH 6.6-7.4). It was therefore concluded that the lack of $[^3]$H GDP binding to skeletal muscle mitochondria is independent of pH.

Figure 6.2.5 shows an overall comparison of the $[^3]$H GDP binding curves and parameters to mitochondria isolated from tissues known to express UCP’s. Figure 6.2.5 shows the saturation binding analysis of $[^3]$H GDP in BAT mitochondria isolated from rats acclimated to the cold for 28 days. The data for BAT mitochondria clearly shows saturation of binding up to 6 µM $[^3]$H GDP. The $K_D$ and $B_{MAX}$ values obtained were $0.7 \pm 0.3$ µM (n=3) and $1640 \pm 204$ pmoles bound/mg protein (n=3) respectively (mean ± S.E.M. of 3 separate experiments, each experiment performed in triplicate). $[^3]$H GDP binding to skeletal muscle and liver mitochondria isolated from fed or fasted rats were non-saturable. Therefore, determination of the $B_{MAX}$ and $K_D$ values were unattainable. It was therefore concluded that $[^3]$H GDP binding to skeletal muscle and liver mitochondria was of low-affinity.

A possible reason for the lack of saturable GDP binding in skeletal muscle mitochondria may be due to masking. Masking has been previously shown to exist in BAT mitochondria containing UCP 1 (Huang & Klingenberg, 1995; Gribskov et al., 1986). Masking is the term used to describe the situation when some or all of the nucleotide binding sites for UCP’s are already occupied by residual bound purine nucleotides prior to performing the binding assay. The purine nucleotide binding sites in BAT mitochondria has been shown to be masked in rats kept at room temperature but unmasked in BAT mitochondria isolated from cold-acclimated rats. As the skeletal muscle mitochondria used in this study are isolated from rats kept at room temperature, it is possible to infer that like UCP 1, UCP 3’s purine nucleotide binding site might be masked, which therefore may account for the lack of saturable binding we see in figure 6.2.4. Huang & Klingenberg, (1995) showed that isolated mitochondria could be unmasked by treating the mitochondria with Dowex (a 21K anion exchanger) at pH 8 (see section 2.22; chapter 2). Using BAT mitochondria isolated from rats kept at room temperature as a control, this study sought to show
Figure 6.2.5: Overall comparison of $[^3]$H GDP binding parameters in BAT mitochondria isolated from cold-acclimated rats, skeletal muscle and liver mitochondria isolated from fed and fasted rats.

Mitochondria were isolated as described in section 2.8. Mitochondria (50 μg) were incubated with $[^3]$H GDP (11.0 Ci/mmol; 0.1-6.0 μM) and $[^14]$C] sucrose (250 μCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5 mM) as described in section 2.22. Specific binding was calculated from the difference between total and non-specific binding. Data represents the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate. Absence of error bars indicates the errors were smaller then the size of the symbol. $K_D$ and $B_{MAX}$ values were obtained for BAT mitochondria by plotting the bound against free ligand values, by non-weighted, non-linear regression to an equation describing a rectangular hyperbola using the computer program Sigma plot. The lines represent best fit.

The $K_D$ and $B_{MAX}$ values obtained for cold-acclimated BAT mitochondria were 0.7 ± 0.3 μM and 1640 ± 204 pmoles bound/mg protein respectively. Determination of the binding parameters $K_D$ and $B_{MAX}$ for skeletal muscle and liver mitochondria were unattainable, as the specific binding was not found to be saturable.
that Dowex-treatment can indeed unmask BAT mitochondria isolated from rats kept at room temperature. This study also sought to investigate whether UCP 3’s purine nucleotide binding site in skeletal muscle mitochondria isolated from rats kept at room temperature was also masked.

Figure 6.2.6 shows saturable $[^3]$H GDP binding to non-Dowex and Dowex-treated BAT mitochondria isolated from rats kept at room temperature. Unmasking, resulting from Dowex-treatment, manifests itself as an increase in $B_{\text{max}}$. The maximal binding capacity of Dowex-treated BAT mitochondria (394 ± 25 pmoles bound/mg protein) increases significantly (P=0.048*; n=3) when compared to non-Dowex treated BAT mitochondria (294 ± 29 pmoles bound/mg protein). Our results therefore show that Dowex unmasks BAT mitochondria by increasing the number of available purine nucleotide binding sites. Dowex-treatment had no effect on the $K_D$ value. $K_D$ values of 0.6 ± 0.2 μM (n=3) and 0.7 ± 0.2 μM (n=3) were obtained for non-Dowex and Dowex-treated BAT mitochondria respectively. Taken together, our results indicate that Dowex-treatment increases the maximal number of binding sites whilst maintaining $[^3]$H GDP’s affinity for the purine nucleotide binding site.

As a negative control, $[^3]$H GDP binding to Dowex-treated liver mitochondria was also investigated. Figure 6.2.7 shows that $[^3]$H GDP binding to Dowex-treated liver mitochondria was of low-affinity. Consequently, determination of the binding parameters, $K_D$ and $B_{\text{max}}$, were unattainable as the specific binding was not found to be saturable. Similarly, $[^3]$H GDP binding to Dowex-treated liver mitochondria isolated from fasted rats was of low-affinity (results not shown).

Figure 6.2.8 shows $[^3]$H GDP binding to Dowex-treated skeletal muscle mitochondria isolated from fed and fasted rats to be of low-affinity with no saturable binding up to 6μM $[^3]$H GDP. Consequently, $K_D$ and $B_{\text{max}}$ values could not be accurately determined.

Figure 6.2.9 shows saturable $[^3]$H GDP binding to Dowex-treated thymus mitochondria isolated from fed and fasted rats. The data clearly shows saturable binding up to 6μM $[^3]$H GDP. The data was fitted to an equation describing a
Figure 6.2.6: Dowex –treatment unmasks UCP 1 in BAT mitochondria by increasing the maximal binding parameter (B_{MAX}) significantly but not the K_{D}.

BAT mitochondria were isolated from room temperature rats as described in section 2.8. BAT mitochondria (2 mg/ml) were incubated with and without Dowex (120 mg/ml) prior to [^{3}H] GDP binding assay as described in section 2.22. BAT mitochondria (50μg) were then incubated with [^{3}H] GDP (12.3 Ci/mmol; 0.1-6.0 μM,) and [^{14}C] sucrose (250 μCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5 mM) as described in section 2.22. Specific binding was calculated from the difference between total and non-specific binding.

The values for specific binding represent the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate. Absence of error bars indicated the errors were smaller then the size of the symbol. K_{D} and B_{MAX} values were obtained by fitting the data to an equation describing a rectangular hyperbola using the computer program Sigma plot. The lines represent best fit.

B_{MAX} values obtained were 294 ± 29 and 394 ± 25* (P=0.048; n=3) pmoles bound/mg protein and K_{D} values of 0.6 ± 0.2 μM and 0.7 ± 0.2 μM for non-Dowex and Dowex-treated BAT mitochondria respectively.
Figure 6.2.7: Low-affinity $[^3]$H GDP binding to Dowex-treated Liver mitochondria.

Liver mitochondria were isolated from fed rats as described in section 2.8. Liver mitochondria (2 mg/ml) were incubated with Dowex (120 mg/ml) for 1 hour at room temperature prior to $[^3]$H GDP binding assay as described in section 2.21. Liver mitochondria (50 μg) were then incubated with $[^3]$H GDP (12.3 Ci/mmol; 0.1-6.0 μM) and $[^14]$C sucrose (250 μCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5 mM) as described in section 2.22. Specific binding was calculated from the difference between total and non-specific binding. Attempts to determine $K_d$ and $B_{max}$ values were achieved by fitting the data to an equation describing a rectangular hyperbola using the computer program Sigma plot. However, determination of the binding parameters $K_d$ and $B_{max}$ were unattainable, as the specific binding was not found to be saturable. Data is expressed as the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate. Absence of error bars indicates the errors were smaller than the size of the symbol.
Figure 6.2.8: Low-affinity $[^3]$H GDP binding to Dowex-treated Skeletal Muscle mitochondria isolated from fed and fasted rats.

Skeletal muscle mitochondria were isolated from fed and fasted rats as described in section 2.8. Skeletal muscle mitochondria (2 mg/ml) were incubated with Dowex (120 mg/ml) for 1 hour at room temperature prior to $[^3]$H GDP binding assay as described in section 2.21. Skeletal muscle mitochondria (50 μg) were then incubated with $[^3]$H GDP (12.3 Ci/mmol; 0.1-6.0 μM) and $[^14]$C sucrose (250 μCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5 mM) as described in section 2.22. Specific binding was calculated from the difference between total and non-specific binding.

Attempts to determine $K_D$ and $B_{MAX}$ values were achieved by fitting the data to an equation describing a rectangular hyperbola using the computer program Sigma plot. However, determination of the binding parameters $K_D$ and $B_{MAX}$ were unattainable, as the specific binding was not found to be saturable. Data is expressed as the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate. Absence of error bars indicates the errors were smaller then the size of the symbol.
Figure 6.2.9: Saturable $[^3]$H GDP binding to Dowex-treated Thymus mitochondria isolated from fed and fasted rats.

Thymus mitochondria were isolated from fed and fasted rats as described in section 2.8. Thymus mitochondria (2 mg/ml) were incubated with Dowex (120 mg/ml) prior to $[^3]$H GDP binding assay as described in section 2.21. Thymus mitochondria (50 µg) were then incubated with $[^3]$H GDP (12.3Ci/mm; 0.1-6.0 µM) and $[^14]$C sucrose (250 µCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5mM) as described in section 2.22. Specific binding was calculated from the difference between total and non-specific binding.

The values for specific binding represent the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate. Absence of error bars indicates the errors were smaller than the size of the symbol. $K_D$ and $B_{MAX}$ values were obtained by fitting the data to an equation describing a rectangular hyperbola using the computer program Sigma plot. The lines represent best fit.

$B_{MAX}$ values obtained were 290± 46 (n=3) and 222 ± 23 (n=3) pmoles bound/mg protein and $K_D$ values of 0.7 ± 0.4 µM (n=3) and 0.9 ± 0.3 µM (n=3) for thymus mitochondria isolated from fed and fasted rats respectively. No significant differences in the $B_{MAX}$ and $K_D$ values were observed between the fed and fasted states.
rectangular hyperbola, using non-weighted, non-linear least square regression analysis. Non-specific, non-saturable binding was determined in the presence of unlabelled GDP. The $K_D$ values determined were $0.7 \pm 0.4 \mu M$ (n=3) and $0.9 \pm 0.3 \mu M$ (n=3) for Dowex-treated thymus mitochondria isolated from fed and fasted rats respectively. $B_{MAX}$ values determined for Dowex-treated thymus mitochondria isolated from fed rats ($290 \pm 46$ pmoles bound/mg protein; n=3) and fasted rats ($222 \pm 23$ pmoles bound/mg protein; n=3) were also calculated. These values are expressed as mean $\pm$ S.E.M. of at least 3 experiments, each experiment performed in triplicate. No significant difference in the $K_D$ and $B_{MAX}$ values were observed between fed and fasted states ($P<1.0$).

Figure 6.2.10 shows $[^3H]$ GDP binding to Dowex-treated spleen mitochondria, isolated from fed and fasted rats, to be of low affinity. No saturable binding was observed up to $6\mu M$ $[^3H]$ GDP. Consequently, determination of the binding parameters, $K_D$ and $B_{MAX}$, were unattainable.

Figure 6.2.11 shows an overall comparison of the $[^3H]$ GDP binding parameters to Dowex-treated mitochondria isolated from tissues known to constitutively express UCP's. Figure 6.2.11 shows saturable $[^3H]$ GDP binding to Dowex-treated BAT and thymus mitochondria isolated from rats kept at room temperature. The data clearly shows saturable binding up to $6 \mu M$ $[^3H]$ GDP. The data was fitted to an equation describing a rectangular hyperbola, using non-weighted, non-linear least square regression analysis. Dowex-treated thymus mitochondria containing UCP 1 bind GDP in a saturable manner with similar binding parameters obtained with Dowex-treated BAT mitochondria. The $K_D$ values determined were $0.7 \pm 0.4 \mu M$ (n=3) and $0.7 \pm 0.2 \mu M$ (n=3) for Dowex-treated thymus and BAT mitochondria respectively. The $B_{MAX}$ values obtained were $290 \pm 46$ pmoles bound/mg protein (n=3) and $394 \pm 25$ pmoles bound/mg protein (n=3) for Dowex-treated thymus and BAT mitochondria respectively. Dowex-treated skeletal muscle, spleen and liver mitochondria isolated from fed and fasted (not shown in figure 6.2.11) rats shows $[^3H]$ GDP binding to be of low-affinity, with no saturable binding up to concentrations of $6\mu M$ $[^3H]$ GDP.
Spleen mitochondria were isolated from fed and fasted rats as described in section 2.8. Spleen mitochondria (2 mg/ml) were incubated with Dowex (120 mg/ml) for 1 hour at room temperature prior to \[^3\text{H}\] GDP binding assay as described in section 2.21. Spleen mitochondria (50 μg) were then incubated with \[^3\text{H}\] GDP (12.3 Ci/mmol; 0.1-6.0 μM) and \[^1\text{C}\] sucrose (250 μCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5 mM) as described in section 2.22. Specific binding was calculated from the difference between total and non-specific binding. Attempts to determine \(K_d\) and \(B_{\text{MAX}}\) values were achieved by fitting the data to an equation describing a rectangular hyperbola using the computer program Sigma plot. However, determination of the binding parameters \(K_d\) and \(B_{\text{MAX}}\) were unattainable, as the specific binding was not found to be saturable. Data is expressed as the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate. Absence of error bars indicates the errors were smaller than the size of the symbol.
Figure 6.2.11: Overall comparison $[^3]$H GDP binding parameters to Dowex-treated mitochondria expressing UCP’s.

Mitochondria were isolated as described in section 2.8. Mitochondria (2 mg/ml) were incubated with Dowex (120 mg/ml) for 1 hour at room temperature as described in section 2.21 prior to $[^3]$H GDP binding assay. Mitochondria (50 μg) were then incubated with $[^3]$H GDP (11.0Ci/mmol; 12.3Ci/mmol; (0.1-6.0 μM) and $[^4]$C sucrose (250 μCi/ml) for 15 minutes at 37°C in the absence (total binding) and presence (non-specific binding) of unlabelled GDP (1.5 mM) as described in section 2.22. Specific binding was calculated from the difference between total and non-specific binding.

The values for specific binding represent the mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate. Absence of error bars indicates the errors were smaller than the size of the symbol. $K_d$ and $B_{max}$ values were obtained by plotting the bound against free ligand values, by non-weighted, non-linear regression to an equation describing a rectangular hyperbola using the computer program Sigma plot. The lines represent best fit.

The $K_d$ and $B_{max}$ values obtained for BAT and thymus mitochondria were 0.7 ± 0.2 μM (n=3); 0.8±0.4 μM (n=3) and 394 ± 25 (n=3); 298 ± 47 (n=3) pmoles bound/mg protein respectively. Determination of the binding parameters $K_d$ and $B_{max}$ for Dowex-treated skeletal muscle, spleen and liver mitochondria were unattainable as the specific binding was not found to be saturable.
Nucleotide binding has been key to identifying and isolating UCP 1 from BAT mitochondria (Heaton et al., 1978; Lin & Klingenberg, 1980). UCP 1 accepts with strong preference the purine ribose di- and tri-nucleotides: GDP, GTP, ATP and ADP (Klingenberg, 1988). Our study shows BAT mitochondria, isolated from rats acclimated to the cold for 28 days, bind GDP with $K_D$ (0.7 ± 0.3 μM) and $B_{MAX}$ (1640 ± 204 pmoles bound/mg protein) binding parameters that are in close accordance with those obtained by other authors in the literature (Sundin & Cannon, 1980; Nedergaard & Cannon, 1985; Sundin et al., 1997). Therefore, BAT mitochondria isolated from cold-acclimated rats act as a positive control tissue for the GDP binding assay employed in this study.

Our results also show that $[^{3}H]$ GDP binding to liver mitochondria is of low-affinity, with no saturable binding up to 6 μM $[^{3}H]$ GDP. Non-saturable GDP binding in liver mitochondria is to be expected, as UCP’s are not constitutively expressed in liver mitochondria (Boss et al., 1997; Fluery et al., 1997; Figure 5.2.2, 5.2.3 & 5.2.4; chapter 5). Therefore, unlike BAT, liver mitochondria act as a negative control tissue for the GDP binding assay used in this study.

One of the main aims of this chapter was to investigate whether mitochondria containing UCP 2 and UCP 3 could bind purine nucleotides, akin to mitochondria containing UCP 1. Therefore, having set up a GDP binding assay that is sensitive for UCP 1, we sought to investigate $[^{3}H]$ GDP binding to rat skeletal muscle mitochondria known to express UCP 3 (Figure 5.2.4 & 5.2.5; chapter 5; Cadenas et al., 1999; Ježek et al., 1999; Gong et al., 2000; Li et al., 2000; Zhou et al., 2000; Giacobino et al., 2001; Cadenas et al., 2002; Harper et al., 2002; Cunningham et al., 2003). However, $[^{3}H]$ GDP binding to skeletal muscle mitochondria was of low-affinity, with no saturable binding up to 6 μM $[^{3}H]$ GDP. Starvation (48-hour) has been shown to increase UCP 3 protein expression 2-3 fold in rat skeletal muscle mitochondria (Wiegle et al., 1998; Cadenas et al., 1999; Samec et al., 1999; Moreno et al., 2003). Therefore, if UCP 3 is a GDP binding protein one might expect to observe an increase in UCP 3’s binding capacity for $[^{3}H]$ GDP in skeletal muscle mitochondria isolated from fasted rats. Our study shows that GDP binding to skeletal
muscle mitochondria isolated from fasted rats was of low-affinity thereby implying that under our binding assay conditions, UCP 3 does not bind purine nucleotides. To date, no other research group have published data showing \[^3\text{H}\] GDP binding to isolated mitochondria containing UCP 2 or UCP 3.

The lack of saturable GDP binding to UCP 3 containing skeletal muscle mitochondria may have resulted from a lack of sensitivity in the GDP binding assay for UCP 3. In order to further extenuate the possibility of obtaining saturable binding to UCP 3, \[^3\text{H}\] GDP binding was performed under various assay conditions. Temperature is an important determinant in optimizing binding assay conditions. \[^3\text{H}\] GDP binding was therefore performed over a range of temperatures using freeze-thawed BAT mitochondria, isolated from cold-acclimated rats. Subtle differences in saturable binding parameters were observed in freeze-thawed BAT mitochondria under different temperature conditions (Table 6.2). Temperature had no effect on the \(B_{\text{MAX}}\) values (within error) in freeze-thawed BAT mitochondria. However, the affinity for \[^3\text{H}\] GDP binding was lower under assay conditions of low temperatures. \[^3\text{H}\] GDP binding was found to be temperature dependent, revealing that although the \(B_{\text{MAX}}\) values remained unchanged, differences in binding affinity were noted. It seems at room temperature (21°C) and 37°C, a tighter fit or a more favorable conformation exists for GDP binding to BAT mitochondria, compared to lower temperatures (0°C and 10°C). Taken together, our results indicate that lowering the temperature did not improve the sensitivity of our assay. In fact, all BAT mitochondria binding assays have been performed at room temperature (21°C) (Sundin & Cannon, 1980; Nedergaard & Cannon, 1985; Gribskov et al., 1986; Swick & Swick, 1986; Sundin et al., 1987; Milner et al., 1988; Peachey et al., 1988) or at 37°C (Scarpace et al., 1991).

One of the most remarkable features of nucleotide binding to UCP 1 is its strong pH dependence (Klingenberg & Huang, 1999). The affinity for purine and pyrimidine nucleotides decrease with increasing pH, notably above 7.2 (Klingenberg & Huang, 1999). To ensure that pH was not a factor in the non-saturable binding observed with UCP 3 containing skeletal muscle mitochondria, we sought to investigate if \[^3\text{H}\] GDP binding was pH sensitive (Table 6.3). However, \[^3\text{H}\] GDP
binding to skeletal muscle mitochondria remained non-saturable (NS) over a range of pH's.

Our results show that Dowex-treatment of BAT mitochondria isolated from room temperature rats significantly increases the $B_{\text{MAX}}$ value 1.3-fold when compared to non-Dowex treated BAT mitochondria (Figure 6.2.6). The increase in the $B_{\text{MAX}}$ value as a result of Dowex-treatment, confirms what is already known that the purine nucleotide binding site in BAT mitochondria isolated from room temperature rats is masked. The affinity of the binding site for GDP would appear to be unaffected as the $K_D$ values obtained for non Dowex-treated and Dowex-treated BAT mitochondria were very similar (0.6 ± 0.2 µM (n=3) and 0.7 ± 0.2 µM (n=3) respectively). The binding parameters, $B_{\text{MAX}}$ and $K_{D}$ values, shown in this study correlate nicely with the literature (Nedergaard & Cannon, 1985; Gribskov et al., 1986; Peachey et al., 1988). The observation that Dowex-treatment involves changes in the number of available binding sites rather than changes in the affinity of the binding site for GDP is also in accordance with previous studies in the literature (Peachey et al., 1988; Klingenberg & Huang, 1999).

Clearly Dowex-treatment maximizes the possibility of detecting GDP binding to UCP 1 containing mitochondria (Figure 1.9, chapter 1). The question was whether Dowex-treatment of mitochondria constitutively expressing UCP 2 and UCP 3 could bind GDP.

Dowex-treated skeletal muscle mitochondria isolated from fed and fasted (48-hour starvation) rats show [$^3$H] GDP binding to be of low-affinity, with no saturable binding up to 6 µM [$^3$H] GDP. Furthermore, our results indicate that the lack of saturable GDP binding to skeletal muscle mitochondria is not due to the purine nucleotide binding site being masked. Therefore, it is safe to conclude that UCP 3 containing mitochondria do not bind GDP in a saturable fashion under the conditions used in this study. To date, no other study has investigated GDP binding to UCP 3 containing skeletal muscle mitochondria.

The study presented here has subsequently shown the novel finding that UCP 3 transcript and protein is constitutively expressed in spleen mitochondria. Other research groups have shown that spleen mitochondria express UCP 2 protein (Richard et al., 1999; Pecqueur et al., 2001). Starvation does not induce an increase
in UCP 2 (Pecqueur et al., 2001) or UCP 3 (Figure 5.2.10; chapter 5) protein expression in spleen mitochondria, unlike in skeletal muscle mitochondria. Therefore we sought to investigate whether UCP 2 or UCP 3 would bind purine nucleotides in spleen mitochondria isolated from fed and fasted rats. However, [3H] GDP binding to Dowex-treated spleen mitochondria is of low affinity, with no saturation of binding up to 6μM GDP. The lack of GDP binding to spleen mitochondria, known to contain UCP 2 and UCP 3 again implies that UCP 2 and UCP 3 do not bind purine nucleotides in their native state. No other research group has investigated GDP binding to isolated spleen mitochondria.

Determination of the binding parameters, K_D and B_MAX, for Dowex-treated liver mitochondria were unattainable presumably because there are no UCP’s present in liver mitochondria (Boss et al., 1997; Fluery et al., 1997; Gong et al., 1997; Figures 5.2.2, 5.2.3 & 5.2.4; chapter 5).

One of the exciting findings in this study is that I have shown that UCP 1 transcript and protein are present in rat thymus and thymus mitochondria respectively (Figures 3.2.4; chapter 3 & Figure 4.2.5; chapter 4). As UCP 1 is a GDP-binding protein in BAT, the existence of UCP 1 protein in thymus mitochondria implied that we might expect to see saturable GDP binding. Using thymus mitochondria isolated and Dowex-treated from fed and fasted rats (to maximize the possibility of detecting binding), we were able to show [3H] GDP binding to be saturable, with binding parameters, K_D (0.7 ± 0.4 and 0.9 ± 0.3 μM; n=3) and B_MAX (290 ± 46 and 222 ± 23 pmoles bound/mg protein; n=3), similar between fed and fasted states. The fact that no significant difference (P<1.0) was observed between K_D and B_MAX values in fed and fasted states correlates with the immunoblotting data previously shown in this study (Figure 4.2.5, chapter 4). The binding parameters obtained for Dowex-treated thymus mitochondria isolated from fed rats are in close accordance with the binding parameters obtained for Dowex-treated BAT mitochondria (Figure 6.2.6).

The fact that UCP 2 (Nègre-Salvayre et al., 1997; Krauss et al., 2002) and UCP 3 (Figure 3.2.5; chapter 3 & Figure 5.2.8; chapter 5) transcript and protein are expressed in thymus and thymus mitochondria respectively may imply that these UCP’s can bind GDP when expressed in thymus mitochondria. Nègre-Salvayre et al.
(1997) has shown that pre-incubation of thymus mitochondrial fractions with GDP, an inhibitor of UCP 1 induced a rise in mitochondrial membrane potential and hydrogen peroxide production (H$_2$O$_2$). Up to now, UCP 2 has been the only UCP associated with thymus and therefore Nègre-Salvayre et al. (1997) concluded that UCP 2 is GDP sensitive and that UCP 2 is a regulator of mitochondrial H$_2$O$_2$ production. These results obtained by Nègre-Salvayre et al. (1997) may imply that UCP 2 is associated with the saturable GDP binding in thymus mitochondria. However, although such a role for UCP 2 may exist, the conclusion of Nègre-Salvayre et al. (1997) remains questionable since GDP binding and inhibition of UCP 2 activity by GDP in isolated mitochondria have yet to be adequately demonstrated. Our study has also shown that UCP 3 transcript and protein is expressed in human thymus and rat thymus mitochondria (Figure 3.2.5; chapter 3 & Figure 5.2.8; chapter 5 respectively). Our results have also shown that starvation induces a 1.5-fold increase in UCP 3 protein expression, whilst having no effect on UCP 1 protein expression in thymus mitochondria. If the GDP binding in thymus mitochondria was associated with UCP 3, one might expect to see an increase in [$^3$H] GDP binding in thymus mitochondria isolated from fasted rats. However, our results show that [$^3$H] GDP binding to Dowex-treated thymus mitochondria isolated from fasted rats showed a slight decrease (within error) in the $B_{MAX}$, whilst having no effect on the $K_D$. This scenario is indicative that GDP binding in thymus mitochondria is not associated with UCP 3. Taken together, these results strongly imply that the binding parameters obtained for thymus mitochondria are due to the presence of UCP 1 and not UCP 2 or UCP 3.

Chemical modification and site-directed mutagenesis studies have shown that the amino acids involved in purine nucleotide binding to UCP 1 are conserved in UCP 2 and UCP 3. Similarly, E.coli and heterologous yeast expression systems have shown GDP binding UCP 2 and UCP 3 (Jekabsons et al., 2002; Zackova et al., 2003). Jekabsons et al. (2002) showed that UCP 2 expressed in bacterial inclusion bodies and solubilised bind N-methylanthraniloyl-tagged (MANT) purine nucleoside di- and tri-phosphates with low micromolar affinity. Zackova et al. (2003) showed that [$^3$H] GTP binding to isolated E.coli or yeast-expressing UCP 2 or UCP 3 exhibit high affinity binding, similar to UCP 1. Taken together, circumstantial evidence has
thus shown that UCP 2 and UCP 3 should bind purine nucleotides, akin to UCP 1. However, we have shown GDP binding to UCP 2 and UCP 3 containing mitochondria to be non-saturable. In mitochondria, there maybe other proteins regulating UCP 2 and UCP 3’s binding capabilities and therefore this may account for the lack of binding observed in our study. In contrast, Jekabsons et al. (2002) and Zackova et al. (2003) observed binding to isolated pure UCP 2 and UCP 3 protein, not UCP 2 or UCP 3-containing mitochondria.

[6.4] Conclusion

Our results confirm that UCP 1 in BAT mitochondria isolated from rats acclimated to the cold for 28 days bind GDP with binding parameters, $K_D$ and $B_{\text{MAX}}$ values very similar to those in the literature. Our findings also confirm that the purine nucleotide binding site in BAT mitochondria isolated from rats kept at room temperature is masked and that Dowex-treating the BAT mitochondria results in the binding site being unmasked.

Taken together, all our binding data shows no saturable GDP binding to mitochondria known to contain UCP 2 and UCP 3. These results may indicate that UCP 2 and UCP 3 do not bind purine nucleotides. The lack of saturable GDP binding to mitochondria known to constitutively express UCP 2 and UCP 3 is not due to the temperature of the assay, pH dependence or masking of the purine nucleotide binding site. The possibility cannot of course be excluded that a purine nucleotide binding site exists on UCP 2 and UCP 3 with purine nucleotide selectivity or affinity that is markedly different from that of UCP 1. Similarly, UCP 2 and UCP 3 may only bind purine nucleotides in the presence of activators such as coenzyme Q, superoxide or hydroxynonenals.

This study has also shown the novel finding that thymus mitochondria have a UCP 1 dependent GDP binding site, with binding parameters, $K_D$ and $B_{\text{MAX}}$ values, very similar to those obtained for BAT mitochondria. The presence of a functional UCP 1 protein in thymus mitochondria has unequivocally exposed a novel route for further research into thymus bioenergetics and function (see general discussion chapter).
Chapter 7

Effects of fatty-acids and purine nucleotides on UCP function
Chapter 7
Effects of fatty-acids and purine nucleotides on UCP function

[7.1] Introduction

In brown adipocytes, the mitochondria are equipped with a high oxidative capacity and are uncoupled due to the presence of UCP 1 in cold-acclimated animals (Nedergaard et al., 2001). BAT mitochondria is responsible for nonshivering theremogenesis (Ricquier & Bouillaud, 1986). UCP 1 represents 12-15% of the mitochondrial inner membrane protein of BAT mitochondria from cold-acclimated hamsters or rats (Lin & Klingenberg, 1980; Huang, 2003). The uncoupling activity of UCP 1 in brown adipose tissue (BAT) is regulated by purine nucleotides and fatty-acids (Nicholls, 2001). Although the inhibition by nucleotides is well established (see chapter 6), the mechanism by which fatty-acids activate UCP 1 remains controversial. The mechanism of uncoupling by UCP 1 has devolved into two models; the fatty-acid protonophore model (Garlid et al., 1996) and the proton buffering model (Winkler & Klingenberg, 1994), both of which have been discussed in detail in Chapter 1 (section 1.10). BAT mitochondria are known to be more sensitive to fatty-acid uncoupling than mitochondria from other tissues, a property that has been assigned to the presence of UCP 1 (González-Barroso et al., 1998). Medium to long-chain fatty-acids are essential for maximal UCP 1 activity (Klingenberg & Huang, 1999). UCP 1 activity can also be fully inhibited by purine nucleoside di- and tri- phosphates such as GDP (Stuart et al., 2001). Physiologically, the fact that UCP 1’s activity can be “turned on” and “turned off” by fatty-acids (palmitate) and GDP, respectively, may be important in allowing BAT to respond to acute changes in the animal’s need to produce heat. Experimentally, purine nucleotides and fatty-acids are useful in allowing the measurement of UCP 1 activity, and in allowing a distinction to be made between the UCP 1 proton conductance pathway and other pathway(s) of mitochondrial inner membrane proton conductance. The purine nucleotide inhibition and fatty-acid activation of UCP 1 activity in BAT mitochondria, has become an interesting paradigm for the study of the other UCP homologues.
The sequence similarity of UCP 2 and UCP 3 to UCP 1 raised the possibility that proton permeability of the mitochondrial inner membrane in organs other than BAT, could be catalyzed by the activity of UCP 2 and UCP 3. To investigate whether fatty-acids activate UCP 2 and UCP 3’s function, in a similar manner to UCP 1, it was necessary to assume that UCP 2 and UCP 3 are natural uncouplers. There are several lines of evidence to support the hypothesis that UCP 2 and UCP 3 are natural uncouplers. Firstly, UCP 2 and UCP 3 are 59% and 56% homologous to UCP 1 respectively and 71% identical to each other (Lowell, 1999). Based upon the high sequence homology of UCP 2 and UCP 3 with UCP 1, it is predicted that UCP 1, 2 and 3 share a similar uncoupling function.

Circumstantial evidence has also shown that UCP 2 and UCP 3 are uncouplers. It has recently been shown that UCP 2 is expressed in pancreatic β-cells (Shimabukuro et al., 1997; Zhou et al., 1997; Chan et al., 1999), raising the possibility that UCP 2 might influence insulin secretion by regulating the amount of ATP derived from metabolized glucose (Boss et al., 1998b). In support of this view, it was observed that adenovirally mediated expression of UCP 2 in pancreatic islets markedly reduced insulin secretion in response to glucose by isolated islets (Chan et al., 1999). Additionally, researchers have shown that UCP 2 uncouples proton transport in macrophages, thereby postulating a role for UCP 2 as an uncoupler in vivo (Couplan et al., 2002). Krauss et al., (2002) has shown that UCP 2, expressed at endogenous levels, mediates proton leak in intact thymocytes.

Evidence for UCP 3 catalysing proton leak in mitochondria is ambiguous. Mice over-expressing human UCP 3 in their skeletal muscle were found to be hyperphagic and leaner than controls, consistent with a uncoupling function and a role in basal metabolism. Skeletal muscle mitochondria isolated from transgenic mice, over-expressing human UCP 3, show a 2-3-fold increase in proton conductance, compared to control mice (Cadenas et al., 2000). More compelling evidence suggesting that UCP 3 is an uncoupling protein has came from two recent studies using UCP 3 knockout (KO) mice (Gong et al., 2000; Vidal-Puig et al., 2000). Gong et al. (2000) and Vidal-Puig et al. (2000) showed that skeletal muscle mitochondria, isolated from UCP 3 KO mice are more coupled (i.e. proton leak is reduced). Furthermore, UCP 3 KO mice had no obvious phenotype and there was no
difference in whole body metabolic rate compared to wild-type animals, suggesting no role in determining basal metabolic rate. Ectasy induced a UCP 3 dependent thermogenenicity in mouse skeletal muscle (Mills et al., 2003). In fact, there is an inducible (GDP-sensitive) uncoupling of skeletal muscle mitochondria associated with UCP 3 under conditions of continuous intramitochondrial and extramitochondrial superoxide production (Echtay et al., 2002 & 2002a; Echtay et al., 2003; Murphy et al., 2003).

Despite this, other researchers have shown a lack of uncoupling activity in mitochondria constitutively expressing UCP 2 and UCP 3 (Cadenas et al., 1999; Jekabsons et al., 1999; Matthias et al., 1999; Cadenas et al., 2000; Nedergaard et al., 2001; Couplan et al., 2002; Cresenzo et al., 2003).

Most of the direct evidence to date showing that UCP 2 and UCP 3 are uncoupling proteins, has come from studies using reconstituted yeast and E.coli UCP expression systems. UCP 2 and UCP 3 have been shown to decrease mitochondrial membrane potential and increase State 4 oxygen consumption rates in yeast mitochondria expressing UCP’s, thus indicating that UCP 2 and UCP 3 can uncouple (Fluery et al., 1997; Boss et al., 1998; Hagen et al., 1999; Hinz et al., 1999; Hinz et al., 1999a; Rial et al., 1999; Stuart et al., 2001). Other research groups have expressed UCP 2 and UCP 3 in E.coli, reconstituted them into proteoliposomes and have shown that UCP 2 and UCP 3 catalyze a purine nucleotide and fatty-acid sensitive proton flux (Jabur et al., 1999 & 2003). Echtay et al. (1999) reconstituted a purine nucleotide sensitive chloride transport from bacterially expressed UCP 1 and UCP 3. Echtay et al. (2001) also showed that UCP 2 and UCP 3 expressed in E.coli inclusion bodies, solublized and reconstituted into liposomes, displayed a coenzyme Q dependent proton transport that was sensitive to both fatty-acids and purine nucleotides. Very recently, Zackova et al. (2003) has shown that yeast and E.coli expressing UCP 2 and UCP 3 are highly active proton transporters, sensitive to both polyunsaturated fatty-acids and purine nucleotides.

This study therefore sought to investigate the effects of purine nucleotides and fatty-acids on proton leak in UCP-containing mitochondria. The assay employed in this study was set up according to the method of González-Barroso et al. (1998). Proton leak was measured indirectly using oxygen consumption rates (state 4), in the
presence of inhibitors of the ATP synthase and the adenine nucleotide carrier. Similar oxygen consumption experiments have been successfully employed to measure UCP 1 catalysed uncoupling in cells and isolated mitochondria (Nicholls & Locke, 1984; Matthias et al., 1999; Nedergaard et al., 2001).

Since this study sought to investigate whether fatty-acids have a stimulatory effect on oxygen consumption rates due to proton leak through UCP’s, it was necessary to take precautions to minimize the presence of free fatty-acids. Firstly, a 2% de-fatted BSA wash was included during the mitochondrial preparations, so as to “mop up” any free fatty-acids released during tissue homogenization. Incorporation of de-fatted BSA to the isolation media has been shown to be efficacious in minimizing the non-UCP dependent uncoupling effects of free fatty-acids (Cadenas et al., 1999; Lanni et al., 1999; Matthias et al., 1999). Secondly, fatty-acid concentrations were buffered using a 1:4 de-fatted BSA:fatty-acid molar ratio, so as to prevent non-specific uncoupling by fatty-acids and to ensure that activation by fatty-acids would be in the nanomolar range. According to González-Barroso et al. (1998), a 1:4 de-fatted BSA:palmitate molar ratio corresponds to a 40nM concentration of free palmitate. Therefore proton leak through UCP’s would be activated by nanomolar amounts of palmitate.

Having detected UCP 1 in thymus mitochondria and having established the existence of GDP-binding in thymus mitochondria, we also wished to determine whether thymus mitochondria had a UCP 1 catalysed uncoupling activity similar to UCP 1 containing BAT mitochondria.

We then sought to investigate the effects of purine nucleotides and fatty-acids on state 4 oxygen consumption rates in skeletal muscle mitochondria, which contain UCP 3, kidney mitochondria, which contain UCP 2 and liver mitochondria, which contain no UCP’s.
[7.2] Results

Figure 7.2.1 shows the steady state oxygen consumption rate (state 4) (232 ± 24 nmoles O/min/mg protein; n=3) in BAT mitochondria, isolated from cold-acclimated rats, is largely uncoupled in the presence of inhibitors of the ATP synthase and adenine nucleotide carrier. The addition of GDP (200 μM) significantly (P=0.003*; n=3) re-couples the state 4 oxygen consumption rate from 232 ± 24 nmoles O/min/mg protein (n=3) to 121 ± 2.2 nmoles O/min/mg protein (n=3), thus indicating the presence of UCP 1. The addition of palmitate (64 μM) (~40 nM free) significantly (P=0.001**; n=3) increased the GDP-coupled state 4 oxygen consumption rate further (250 ± 7 nmoles O/min/mg protein). The addition of GDP significantly (P=0.0052**; n=3) decreased the palmitate-stimulated oxygen consumption rate to 155 ± 2 nmoles O/min/mg protein. The maximal uncoupled oxygen consumption rate attained by 40 nM FCCP was 251 ± 28 nmoles O/min/mg protein.

Figure 7.2.2 shows that the GDP-coupled state 4 oxygen consumption rate in BAT mitochondria, isolated from cold-acclimated rats, are sensitive to long chain fatty-acids and fatty acid analogues. The addition of palmitate (64 μM) to the oxygen electrode chamber significantly (P=0.001*; n=3) increased the GDP-coupled state 4 oxygen consumption rate from 121 ± 2.2 nmoles O/min/mg protein to 250 ± 7 nmoles O/min/mg protein. Similarly, the addition of retinoic acid (64 μM) significantly (P=0.01**; n=3) increased the steady-state oxygen consumption rates from 121 ± 2.2 nmoles O/min/mg protein to 235 ± 25.4 nmoles O/min/mg protein. The addition of a retinoic acid analogue, AM580, had no significant (NS) effect on the GDP-coupled state 4 oxygen consumption rate (105 ± 11 nmoles O/min/mg protein; n=3).

Our study also investigated the effects of purine nucleotides and long chain fatty-acids on state 4 oxygen consumption rates in BAT mitochondria isolated from rats kept at room temperature. Figure 7.2.3 shows the state 4 oxygen consumption rate (175 ± 6 nmoles O/min/mg protein; n=3) in the presence of inhibitors of the adenine nucleotide carrier and the ATP synthase was significantly decreased.
Figure 7.2.1: State 4 oxygen consumption rates in BAT mitochondria, isolated from cold-acclimated rats, are inhibited by purine nucleotides and activated by palmitate.

BAT mitochondria were isolated from rats acclimated to the cold for 28 days as described in section 2.8. BAT mitochondria (0.15 mg/ml) were incubated at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH, pH 7.4, 1 mM EGTA, 16 µM de-fatted BSA, 5 µM rotenone, 1 µg/ml oligomycin, 5 µM atractyloside, (± 200 µM GDP) and 7.5 mM succinate (K⁺-salt) in a pre-calibrated Clarke-type oxygen electrode. Steady-state oxygen consumption rates were then measured (State 4), followed by the addition of 64 µM palmitate (~40 nM free) (Palmitate), GDP (200 µM) and 40 nM FCCP (FCCP). Addition of 200 µM GDP to the initial incubation mixture decreased the State 4 respiration rate significantly (P=0.003*; n=3). The addition of 64 µM palmitate (~40 nM free) increased the State 4 (+GDP) oxygen consumption rates significantly (P=0.0029**; n=3). The addition of GDP significantly (P=0.0052***; n=3) inhibited the palmitate induced oxygen consumption rate. Data is expressed as mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate.
Figure 7.2.2: GDP-coupled State 4 oxygen consumption rates in BAT mitochondria, isolated from cold-acclimated rats, are activated by palmitate and retinoic acid but not by AM580.

BAT mitochondria were isolated from rats acclimated to the cold for 28 days as described in section 2.8. BAT mitochondria (0.15 mg/ml) were incubated at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH, pH 7.4, 1 mM EGTA, 16 μM de-fatted BSA, 5 μM rotenone, 1 μg/ml oligomycin, 5 μM atractyloside, 200μM GDP and 7.5 mM succinate (K+-salt) in a pre-calibrated Clarke-type oxygen electrode. Steady-state oxygen consumption rates were then measured (State 4), followed by the addition of 64 μM palmitate (~40nM free) (Palmitate), 64 μM retinoic acid (Retinoic) and 64 μM AM580 (AM580). Addition of palmitate and retinoic acid increased the State 4 oxygen consumption rate significantly (*; P=0.0029 (n=3); **; P=0.01 (n=3)). AM580, a negative analogue of retinoic acid, had no significant effect on State 4 respiration (NS: non significant). Data is expressed as mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate.
Figure 7.2.3: State 4 oxygen consumption rates in BAT mitochondria, isolated from rats kept at room temperature, are inhibited by purine nucleotides and activated by palmitate.

BAT mitochondria were isolated from rats kept at room temperature as described in section 2.8. BAT mitochondria (1 mg/ml) were incubated at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH, pH 7.4, 1 mM EGTA, 16 μM de-fatted BSA, 5 μM rotenone, 1 μg/ml oligomycin, 5 μM atractyloside, and 7.5 mM succinate (K+-salt) in a pre-calibrated Clarke-type oxygen electrode. Steady-state oxygen consumption rates were then measured (State 4), followed by the addition of 1 mM GDP (GDP), followed by the addition of 64 μM palmitate (~40nM free) (Palmitate), and 40nM FCCP (FCCP). The addition of 1 mM GDP decreased the oxygen consumption rate significantly (P=0.004*; n=3). The GDP-sensitive oxygen consumption rate was significantly increased by the addition of 64 μM palmitate (Palmitate) (P=0.04**; n=3). Data is expressed as mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate.
(P=0.004*; n=3) by the addition of GDP (1mM) (129 ± 5 nmoles O/min/mg protein; n=3). The GDP-sensitive state 4 oxygen consumption rate was significantly increased (P=0.0088**; n=3) by the addition of 64 μM palmitate (~40 nM free) to 190 ± 11 nmoles O/min/mg protein (n=3), close to the maximal uncoupled rate attained by 40 nM FCCP (231 ± 14 nmoles O/min/mg protein).

Figure 7.2.4 shows the effects of GDP and palmitate on state 4 oxygen consumption in thymus mitochondria isolated from rats kept at room temperature. The steady-state oxygen consumption rate (state 4) (64 ± 3 nmoles O/min/mg protein; n=3) obtained for thymus mitochondria was significantly decreased (P=0.004*; n=3) upon addition of GDP (1mM) (34 ± 4 nmoles O/min/mg protein; n=3). This GDP-sensitive state 4 oxygen consumption rate was significantly increased (P=0.04**; n=3) by the addition of palmitate (64 μM) (~40 nM free). Finally, the addition of 40 nM FCCP stimulated the maximal oxygen consumption rate in thymus mitochondria (107 ± 4; n=3).

Figure 7.2.5 shows the effects of GDP and palmitate on state 4 oxygen consumption rates in UCP 3 containing skeletal muscle mitochondria. The skeletal muscle mitochondria were isolated from rats kept at room temperature. The steady-state oxygen consumption rate (State 4) in skeletal muscle mitochondria (101 ± 22 nmoles O/min/mg protein; n=3), in the presence of inhibitors of the ATP synthase and the adenine nucleotide carrier, were insensitive to the addition of GDP (1mM) and palmitate (64 μM), unlike BAT and thymus mitochondria. This insensitivity to purine nucleotides and long chain fatty-acids was not due to poor quality mitochondria preparations as the maximal oxygen consumption rates obtained by the addition of FCCP (40 nM) were 280 ± 16 nmoles O/min/mg protein (n=3).

Figure 7.2.6 shows the effects of GDP and palmitate on state 4 oxygen consumption in kidney mitochondria isolated from rats kept at room temperature. Figure 7.2.6 shows that the state 4 oxygen consumption rate in kidney mitochondria (66 ± 13 nmoles O/min/mg protein; n=3), in the presence of inhibitors of the ATP synthase and the adenine nucleotide carrier, are insensitive to the addition of GDP
Figure 7.2.4: State 4 oxygen consumption rates in thymus mitochondria, isolated from rats kept at room temperature, are inhibited by purine nucleotides and activated by palmitate.

Thymus mitochondria were isolated from rats kept at room temperature as described in section 2.8. Thymus mitochondria (1 mg/ml) were incubated at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH, pH 7.4, 1 mM EGTA, 16 μM de-fatted BSA, 5 μM rotenone, 1 μg/ml oligomycin, 5 μM atractylloside, and 7.5 mM succinate (K⁺-salt) in a pre-calibrated Clarke-type oxygen electrode. Steady-state oxygen consumption rates were then measured (State 4), followed by the addition of 1 mM GDP (GDP), followed by the addition of 64 μM palmitate (~40nM free) (Palmitate), and 40nM FCCP (FCCP). The addition of 1 mM GDP decreased the oxygen consumption rate significantly (P=0.008*; n=3). The GDP-sensitive oxygen consumption rate was significantly increased by the addition of 64 μM palmitate (Palmitate) (P=0.04**; n=3). Data is expressed as mean ± S.E.M of at least 3 independent experiments, each experiment performed in triplicate.
Figure 7.2.5: State 4 oxygen consumption rates in skeletal muscle mitochondria, isolated from rats kept at room temperature, are insensitive to purine nucleotides and fatty-acids (i.e. palmitate).

Skeletal muscle mitochondria were isolated from rats kept at room temperature as described in section 2.8. Skeletal muscle mitochondria (1 mg/ml) were incubated at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH, pH 7.4, 1 mM EGTA, 16 μM de-fatted BSA, 5 μM rotenone, 1 μg/ml oligomycin, 5 μM atractyloside, and 7.5 mM succinate (K⁺-salt) in a pre-calibrated Clarke-type oxygen electrode. Steady-state oxygen consumption rates were then measured (State 4), followed by the addition of 1 mM GDP (GDP), followed by the addition of 64 μM palmitate (~40nM free) (Palmitate), and 40nM FCCP (FCCP). No significant change in the oxygen consumption rate was observed upon addition of GDP (1 mM) or palmitate (64 μM) (NS: non-significant). Data is expressed as mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate.
Figure 7.2.6: State 4 oxygen consumption rates in kidney mitochondria, isolated from rats kept at room temperature, are insensitive to purine nucleotides and fatty-acids (*i.e.* palmitate).

Kidney mitochondria were isolated from rats kept at room temperature as described in section 2.8. Kidney mitochondria (1 mg/ml) were incubated at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH, pH 7.4, 1 mM EGTA, 16 μM de-fatted BSA, 5 μM rotenone, 1 μg/ml oligomycin, 5 μM atractyloside, and 7.5 mM succinate (K⁺-salt) in a pre-calibrated Clarke-type oxygen electrode. Steady-state oxygen consumption rates were then measured (State 4), followed by the addition of 1 mM GDP (GDP), followed by the addition of 64μM palmitate (~40nM free) (Palmitate), and 40nM FCCP (FCCP). No significant change in the oxygen consumption rate was observed upon addition of GDP (1 mM) or palmitate (64 μM) (NS: non-significant). Data is expressed as mean ± S.E.M of at least 3 experiments, each experiment performed in triplicate.
(1 mM) and palmitate (64 μM) (~40 nM free). The lack of inhibition and activation by GDP and palmitate respectively, was not due to poor quality mitochondria as the maximal oxygen consumption rates obtained by FCCP (40 nM) were 226 ± 19 nmoles O/min/mg protein; n=3).

Liver mitochondria do not constitutively express UCP’s and were therefore used as a negative control tissue in this study. Figure 7.2.7 shows the effects of GDP and palmitate on state 4 oxygen consumption in liver mitochondria isolated from rats kept at room temperature. Figure 7.2.7 shows the state 4 oxygen consumption rate in liver mitochondria (40 ± 1.4 nmoles O/min/mg protein; n=3), in the presence of inhibitors of the ATP synthase and the adenine nucleotide carrier, to be insensitive to both GDP (1 mM) and palmitate (64 μM) (~40 nM free). The maximal oxygen consumption rate obtained by the addition of FCCP (40 nM) was 131 ± 12 nmoles O/min/mg protein (n=3).

To check whether the isolated mitochondria used in this study were coupled (intact), the respiratory control ratio’s (RCR’s) were determined. Table 7.1 shows the RCR values for BAT mitochondria isolated from cold-acclimated rats (1.6 ± 0.07; n=3), BAT (1.3 ± 0.04; n=3), thymus (1.7 ± 0.07; n=3), skeletal muscle (2.8 ± 0.5; n=3), kidney (3.2 ± 0.9; n=3) and liver (3.3 ± 0.3; n=3) mitochondria isolated from rats kept at room temperature. In our study, the RCR is defined as the (State 3) uncoupled rate (due to the addition of excess artificial uncoupler, FCCP) divided by the steady-state (State 4) oxygen consumption rate.
Figure 7.2.7: State 4 oxygen consumption rates in liver mitochondria, isolated from rats kept at room temperature, are insensitive to purine nucleotides and fatty-acids (i.e. palmitate).

Liver mitochondria were isolated from rats kept at room temperature as described in section 2.8. Liver mitochondria (1 mg/ml) were incubated at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH, pH 7.4, 1 mM EGTA, 16 μM de-fatted BSA, 5 μM rotenone, 1 μg/ml oligomycin, 5 μM atractyloside, and 7.5 mM succinate (K⁺-salt) in a pre-calibrated Clarke-type oxygen electrode. Steady-state oxygen consumption rates were then measured (State 4), followed by the addition of 1 mM GDP (GDP), followed by the addition of 64 μM palmitate (~40nM free) (Palmitate), and 40nM FCCP (FCCP). No significant change in the oxygen consumption rate was observed upon addition of GDP (1 mM) or palmitate (64 μM) (NS: non-significant). Data is expressed as mean ± S.E.M of at least 3 independent experiments, each experiment performed in triplicate.
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<tr>
<td>BAT</td>
<td>1.3 ± 0.04 (n=3)</td>
</tr>
<tr>
<td>Thymus</td>
<td>1.7 ± 0.07 (n=3)</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>2.8 ± 0.5 (n=3)</td>
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<tr>
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</tr>
<tr>
<td>Liver</td>
<td>3.3 ± 0.3 (n=3)</td>
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Table 7.1: Respiratory control ratios (RCR's) for BAT mitochondria isolated from rats acclimated to the cold for 28 days and for BAT, Thymus, Skeletal muscle, Kidney and Liver mitochondria isolated from rats kept at room temperature.

Mitochondria were isolated from rats kept at room temperature as described in section 2.8. Liver mitochondria (1 mg/ml) were incubated at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH, pH 7.4, 1 mM EGTA, 16 μM de-fatted BSA, 5 μM rotenone, 1 μg/ml oligomycin, 5 μM atractyloside, and 7.5 mM succinate (K⁺-salt) in a pre-calibrated Clarke-type oxygen electrode. Steady-state oxygen consumption rates were then measured (State 4), followed by the addition of 40nM FCCP (FCCP) to stimulate a maximum respiration rate (State 3 uncoupled rate). RCR values were obtained using the formula: State 3 uncoupled/ State 4 respiration rate. Data is expressed as mean ± S.E.M of at least 3 independent experiments, each experiment performed in triplicate.
[7.3] Discussion

In mammalian brown adipose tissue (BAT), thermogenesis is explained by the uncoupling mitochondrial ATP synthesis from respiration. UCP 1 is responsible for this uncoupling as it allows proton re-entry into the matrix, and thus dissipates the mitochondrial proton electrochemical gradient generated by the electron transport chain (Rial et al., 1999). Proton transport by UCP 1 is regulated positively by fatty-acids and negatively by purine nucleotides.

In the study presented here, we show that state 4 oxygen consumption rates in BAT mitochondria, isolated from rats acclimated to the cold for 28 days, are innately uncoupled i.e. show a high state 4 oxygen consumption rate in the absence of ATP synthesis (Figure 7.2.1). Our results showing that freshly prepared BAT mitochondria are uncoupled, correlates well with results obtained by other authors in the literature (Matthias et al., 1999; Nicholls et al., 2001). The ability of GDP to inhibit and nanomolar amounts long chain fatty-acids (i.e. palmitate) to activate the state 4 oxygen consumption rates in BAT mitochondria, respectively, compares favorably with the literature (Matthias et al., 1999; Nicholls et al., 2001).

Data presented in this study has also confirmed that addition of nanomolar amounts of fatty-acids (i.e. palmitate) and fatty-acid analogues (i.e. retinoic acid) to GDP-coupled BAT mitochondria, isolated from rats acclimated to the cold, induces uncoupling close to the maximal uncoupled rate (FCCP) (Figure 7.2.2). This study also shows that AM580, an analogue of retinoic acid, did not increase state 4 oxygen consumption rates in BAT mitochondria isolated from cold-acclimated rats. Our results confirm the findings of González-Barroso et al. (1998) and Rial et al. (1999). González-Barroso et al. (1998) and Rial et al. (1999) has previously shown that palmitic acid and retinoic acid, respectively, activates proton conductance through UCP 1 in BAT mitochondria and yeast mitochondria expressing UCP. Similarly, Rial et al. (1999) has shown that AM580 had no effect on increasing proton conductance in yeast mitochondria expressing UCP’s and we have confirmed this result using our mammalian system.

Our study also investigated the effects of purine nucleotides and fatty-acids on BAT mitochondria isolated from rats kept at room temperature. Our results show the steady-state (State 4) oxygen consumption rates in BAT mitochondria, isolated
from rats kept at room-temperature are sensitive to GDP and activated by nanomolar amounts of fatty-acids (Figure 7.2.3). Our results also demonstrate the steady state (State 4) oxygen consumption rates in BAT mitochondria, isolated from rats kept at room temperature are more coupled (175 ± 6 nmoles O/min/mg protein; n=3), when compared to BAT mitochondria isolated from cold-acclimated rats (232 ± 24 nmoles O/min/mg protein; n=3). The decrease in oxygen consumption (State 4) rates in BAT mitochondria, isolated from rats kept at room temperature, is probably associated with a decrease in UCP 1 protein expression. Western blot analysis, previously used in this study, has shown a significant 2-fold decrease in UCP 1 protein expression in BAT mitochondria isolated from rats kept at room temperature, compared to BAT mitochondria isolated from cold-acclimated rats (Figure 4.2.5; chapter 4).

Having established that thymus mitochondria have a UCP 1 dependent GDP-binding site, it remained to be determined whether thymus mitochondria had a UCP 1 catalysed uncoupling activity similar to which has been observed with UCP 1 containing BAT mitochondria. Our results show that steady state (state 4) oxygen consumption rates in thymus mitochondria, isolated from rats kept at room temperature, are inhibited by GDP and activated by nanomolar amounts of palmitate.

Sequence similarity of UCP 2 and UCP 3 to UCP 1, evidence from heterologous yeast and mammalian cell expression systems and studies using UCP 3 knockout mice have hypothesized a role for UCP 2 and UCP 3 as uncoupling proteins. However, the data presented in this study negate this hypothesis. Under our assay conditions, that demonstrate UCP 1 uncoupling activity in BAT and thymus mitochondria, we show that state 4 oxygen consumption rates in skeletal muscle (UCP 3), kidney (UCP 2) and liver (no UCP's) mitochondria are insensitive to GDP and nanomolar amounts of palmitate. This insensitivity to GDP and fatty-acids are not due to poor quality mitochondrial preparations as indicated by the RCR values (Figure 7.2.8). From this, we can conclude that mitochondria constitutively expressing UCP 2 and UCP 3 are not regulated in the same manner to UCP 1.

The lack of mitochondrial uncoupling activity by UCP 2 and UCP 3 shown in this study corroborates well with other findings in the literature (Cadenas et al., 1999; Jekabsons et al., 1999; Matthias et al., 1999; Cadenas et al., 2000; Nedergaard et al., 2001; Couplan et al., 2002; Cresenzo et al., 2003). However, our results
contrasts with those authors who showed purine nucleotide inhibition to UCP 2 and UCP 3 reconstituted into artificial liposomes either with nM (Echtay et al., 1999) or μM affinity (Jabur et al., 1999).

To date, the only direct evidence to show a GDP-sensitive and fatty-acid activated proton leak in mitochondria constitutively expressing UCP 2 and UCP 3 has been under conditions of continuous intramitochondrial and extramitochondrial superoxide production (Echtay et al., 2002 & 2002a; Echtay et al., 2003; Murphy et al., 2003). Therefore, one may imply that only in situations of continous superoxide production that we would observe a GDP and fatty-acid sensitive uncoupling activity in UCP 2 and UCP 3 containing mitochondria. This of-course will require further investigation.

[7.4] Conclusion

In conclusion, the study presented here has confirmed that proton leak, measured indirectly using the rate of oxygen consumption, in BAT and thymus mitochondria are inhibited by GDP and activated by nanomolar amounts of fatty-acids. Although the oxygen consumption rates for thymus mitochondria are approximately a third that of BAT mitochondria, it is safe to conclude that the GDP inhibition and palmitate-activation of oxygen consumption rates in thymus mitochondria is due to the activity of UCP 1.

On the contrary, no GDP sensitivity or fatty-acid activation of proton leak was detected in skeletal muscle mitochondria, which is known to contain UCP 3, kidney mitochondria, which is known to contain UCP 2 or liver mitochondria, which is known to contain no UCP’s (Figure 5.2.2 & 5.2.3; Ricquier & Bouillaud, 2000). Taken together, these results indicate that either UCP 2 and UCP 3 are not uncouplers in mitochondria or if they do catalyse proton leak, then they are regulated differently to UCP 1.
Chapter 8

Identification of UCP 1 protein in thymus mitochondria

using MALDI-TOF and LC-MS mass spectrometry
Chapter 8
Identification of UCP 1 protein in thymus mitochondria
Using MALDI-TOF and LC-MS mass spectrometry

[8.1] Introduction

In this study, western blot analysis shows the novel finding that UCP 1 protein is present in rat thymus mitochondria. Purine nucleotide binding studies have shown that UCP 1 in thymus mitochondria binds GDP with similar binding parameters (i.e. $K_D$ and $B_{MAX}$ values) to UCP 1 in BAT mitochondria.

The western blot analyses and GDP binding assays led us to believe that there would be enough of the UCP 1 protein expressed in mitochondria of rat thymus to allow its purification. In order to purify UCP 1 from rat thymus mitochondria, we employed methodology used to purify UCP 1 from BAT mitochondria (Lin & Klingenberg, 1980). The success of the purification is dependent on correct identification of UCP 1. To that end, we established a collaboration with Dr. Terry Pearson at the University of Victoria, (Victoria, British Columbia, Canada) who has experience in the application of mass spectrometry to identify proteins.
Results

Figure 8.2.1 shows a colloidal Coomassie Blue G-250 stained one dimensional SDS-PAGE gel of UCP 1 that I purified from rat thymus mitochondria. The colloidal Coomassie blue G250 stained the protein molecular weight markers (Mw) and a predominant ~33 kDa protein band in the purified UCP 1 sample. The ~33 kDa protein band was carefully excised from the SDS-PAGE gel and subjected to in-gel digestion with trypsin, prior to mass spectrometric analysis.

Figure 8.2.2 shows the MALDI-TOF mass spectrum obtained by our collaborators Dr. Terry W. Pearson and Lee R. Haines at the University of Victoria, Canada, for purified UCP 1. After internal calibration, baseline correction and peak selection, 33 peptide masses were selected for mass fingerprinting. Selected peptide masses were submitted to four online search algorithms that use peptide masses to identify proteins from primary sequence databases: MS-Fit (Protein Prospector software package; San Francisco, CA: http://prospector.ucsf.edu/), Mascot (Matrix Science, London, UK: http://www.matrixscience.com/), ProFound (Prowl, Rockefeller University - Proteometric: http://prowl.rockefeller.edu), and PeptideSearch (EMBL Bioanalytical Research Group, Heidelberg, Germany: http://www.mann.embl-heidelberg.de/GroupPages/PageLink/peptidesearch/Services/PeptideSearch/FR_PeptideSearchFormG4.html). Searching of the nonredundant database with the peptide masses, identified mitochondrial brown fat uncoupling protein 1 (UCP 1) from Rattus norvegicus as having the top Mowse score (best database “hit”). Nine tryptic peptides and 15 non-specific peptide matches were identified which provided 77% coverage of the UCP 1 protein (237 of 307 amino acids; Table 8.1). These results unequivocally identified UCP 1 as the predominant protein in the gel band.

To refine the mass spectrometric identification of the purified protein, tandem mass spectrometry (LC-MS/MS) was performed. Peptides were separated by liquid chromatography, gated and fragmented. The fragmented data were used to search the database. The results are shown in Table 8.2 (A). Four peptides (covering 15% of the
Figure 8.2.1 Colloidal Coomassie Blue G-250 stained SDS-PAGE gel of UCP 1 purified from thymus mitochondria.

Figure 8.2.1 (A) UCP1 protein was purified from rat thymus mitochondria and subjected to SDS-PAGE (12% resolving gel) (sections 2.19 & 2.13). The gel was stained with Colloidal Coomassie blue G-250, which yielded a single ~33kDa band. The ~33kDa band was excised and subjected to in-gel digestion with trypsin, prior to mass spectrometric analysis.
Figure 8.2.2: MALDI-TOF mass spectrum obtained for UCP 1 purified from rat thymus mitochondria

Data obtained from Dr. Terry W. Pearson and Lee R. Haines at the University of Victoria, Canada,
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<td>66</td>
<td>80</td>
<td>AKTEGLPKLYSGLPA</td>
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<td>55</td>
<td>74</td>
<td>YKGVLGTITTLAKTEGLPKL</td>
<td>non-specific</td>
</tr>
</tbody>
</table>

Masses without numerical designation were not abundant enough to be seen on the spectra without amplification of the baseline.

† C-terminus of UCP 1

Table 8.1: MALDI-TOF identification of UCP 1 from peptide mass fingerprinting.

Data from Dr. Terry W. Pearson and Lee R. Haines at the University of Victoria, Canada.
<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Mass (Daltons)</th>
<th>Accession Number (NCBI)</th>
<th>Isoelectric point</th>
<th># of peptides</th>
<th>% coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoupling protein 1 (UCP 1)</td>
<td>33,400</td>
<td>P04633</td>
<td>9.2</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Voltage-dependent anion-selective channel protein-1 (VDAC-1)</td>
<td>32,500</td>
<td>Q9Z2L0</td>
<td>8.35</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Voltage-dependent anion-selective channel protein-2 (VDAC-2)</td>
<td>32,300</td>
<td>P81155</td>
<td>7.44</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Carnitine/acylcarnitine carrier protein</td>
<td>33,400</td>
<td>P9752</td>
<td>9.55</td>
<td>3</td>
<td>7</td>
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(B)

<table>
<thead>
<tr>
<th>Residue start-Residue end</th>
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<tbody>
<tr>
<td>40-53</td>
<td>LQIQGEGQASSTIR</td>
</tr>
<tr>
<td>73-83</td>
<td>LYSGLPAGIQR</td>
</tr>
<tr>
<td>84-91</td>
<td>QISFASLR</td>
</tr>
<tr>
<td>162-174</td>
<td>VIATTESLSTLWK</td>
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</table>

Table 8.2 (A) LC-MS/MS identification of proteins using peptide fragmentation data. (B) Sequence of the 4 peptides obtained for UCP 1. These 4 peptides cover 15% of the thermogenin (UCP-1) sequence. Data was obtained from Dr. Terry W. Pearson and Lee R. Haines at the University of Victoria, Canada.
protein) identified rat UCP 1. The four peptides are displayed in Table 8.2 (B). Other peptide sequences were also found identifying three other mitochondrial proteins; the voltage-dependent anion selective channel protein-1 (VDAC-1), the voltage-dependent anion selective channel protein-2 (VDAC-2) and the carnitine/acylcarnitine carrier protein (CAC).
[8.3] Discussion

The detection of UCP 1 in rat thymus mitochondria using western blot analysis and the extent of the saturability of the GDP-binding in thymus mitochondria, suggested to us that sufficient UCP 1 protein exists in thymus mitochondria to attempt its purification. Using procedures for purification of UCP 1 from BAT mitochondria (Lin & Klingenberg, 1980), rat thymus mitochondria yielded a single broad ~33 kDa protein band on a one-dimensional SDS-PAGE gel, indicating the presence of UCP 1 (Figure 8.2.1). Following excision of the purified UCP 1 band from the gel, our collaborators Dr. Terry W. Pearson and Lee R. Haines at the University of Victoria, Canada digested the protein with trypsin prior to peptide mass mapping and tandem mass spectrometry using MALDI-TOF and LC-MS/MS. Mass spectrometry using MALDI-TOF and LC-MS/MS confirmed that the predominant protein in the protein band was rat uncoupling protein 1 (NCBI accession # P04633) (Table 8.1 and 8.2). These data are consistent with both the mRNA and protein detection data and functional data previously shown in this study. Taken together, these results provide unequivocal evidence for the presence of UCP 1 in rat thymus mitochondria. Although peptide mass fingerprinting identified only UCP 1 as the predominant protein in the protein band, LC–MS mass spectrometric analysis revealed peptides from three other proteins, voltage-dependent anion-selective channel protein 1 (VDAC-1), voltage-dependent anion-selective channel 2 (VDAC-2) and mitochondrial carnitine/acylcarnitine carrier protein (Table 8.2). These three proteins have masses of 32.5 kDa, 32.3 kDa and 33.4 kDa, respectively, thus it is not surprising that they run along with UCP 1 (33,458 Da) as a single band on a one-dimensional SDS-PAGE gel. However, the pis of the 4 proteins are all distinct and range from 7.44 to 9.55. This would allow their separation by two-dimensional gel electrophoresis, a procedure with much higher resolution than simple one-dimensional gel electrophoresis.

[8.4] Conclusion

In conclusion, the study presented here has now indisputable evidence that UCP 1 is expressed in rat thymus mitochondria. Although UCP 1’s function in
thymus mitochondria has yet to be elucidated, its discovery has exposed a novel route for research into thymus bioenergetics, metabolism and function.
Chapter 9

General Discussion
Chapter 9
General Discussion

[9.1] UCP 1 in thymus

As mentioned earlier (section 3.1; Chapter 3), RT-PCR is the most sensitive method of RNA detection available (Larrick & Siebert, 1995). RT-PCR detects the expression of specific genes, which have low abundance RNA or are present in limiting amounts of tissue (He et al., 1995). Data from RT-PCR can be obtained on < 1ng of total RNA, compared to the ~10μg RNA required for Northern blotting. He et al. (1995) has also shown that RT-PCR is up to 10,000-fold more sensitive than Northern blotting in detecting gene expression. Using RT-PCR analysis, we have shown the novel finding that rat thymus contains UCP 1 mRNA (Chapter 3). Our novel finding contrasts with the observation of Nègre-Salvayre et al. (1997) who were unable to detect UCP 1 mRNA in rat thymus using Northern blot analysis. The lack of detection by Northern blot analysis suggests that UCP 1 mRNA exists in low abundance in rat thymus (less than several μg’s). Our novel finding has also raised the possibility that UCP 1 may exist in other tissues. Future work will investigate this possibility.

Using Western blot analysis, we have shown the anti-UCP 1 peptide antibodies (Calbiochem and Eurogentec) employed in this study to be sensitive for UCP 1 over UCP 2, UCP 3 and other mitochondrial transporters (Chapter 4). Therefore, we are absolutely confident that these antibodies are only detecting UCP 1 and not UCP 2 or UCP 3. Using these sensitive UCP 1 antibodies, our study has shown the novel finding that UCP 1 protein is expressed in thymus and thymus cell (i.e. thymocyte) mitochondria. Under conditions of acute starvation (48-hour), we could not detect any significant (P<1.0) difference in abundance of UCP 1 protein in thymocyte or thymus mitochondria (Chapter 4). It has previously been shown that UCP 1 protein expression in BAT decreases upon starvation (Rothwell et al., 1984; Trayhurn et al., 1988; Boss et al., 1998; Nedergaard et al., 2001). The fact that starvation decreases UCP 1 expression in BAT, whilst having no effect on UCP 1 expression in thymus, may imply that UCP 1 functions differently in BAT than in
thymus. One obvious research angle will be to investigate UCP 1 protein expression in thymus under various physiological conditions known to induce UCP 1 expression in BAT (e.g. cold-exposure and thyroid treatment).

As UCP 1 is a GDP-binding protein in BAT, the existence of UCP 1 protein in thymus mitochondria implied that we might also expect to observe saturable GDP binding. Using isolated and Dowex-treated (to maximize the possibility of detecting binding) thymus mitochondria from fed and fasted rats, we were able to show [3H] GDP binding to be saturable, with $K_D$ and $B_{MAX}$ values similar to those obtained with BAT mitochondria isolated from rats kept at room temperature (Chapter 6). No significant difference ($P<1.0$) was observed between $K_D$ and $B_{MAX}$ values of thymus mitochondria isolated from fed or fasted rats. This observation correlates well with the immunoblotting data previously described above (Chapter 4). One aspect of future studies will be to investigate whether the UCP 1 purine nucleotide binding site in thymus mitochondria are masked at room temperature, akin to UCP 1 in BAT mitochondria.

In addition, our results show that non-phosphorylating oxygen consumption rates in thymus mitochondria, isolated from rats kept at room temperature, are inhibited by GDP and activated by nanomolar concentrations of palmitate (Chapter 7). Although the oxygen consumption rates for non-Dowex treated thymus mitochondria are approximately a third that of BAT mitochondria, they are GDP inhibitable and palmitate activatable, which we conclude to be due to the activity of UCP 1. Clearly this assay system can be used to seek novel activators and inhibitors of UCP 1's uncoupling activity in rat thymus mitochondria and these compounds may well act as modulators of T-lymphocyte function.

The detection of UCP 1 in rat thymus mitochondria using western blot analysis and the extent of the saturability of the GDP-binding in thymus mitochondria, suggested to us that sufficient UCP 1 protein exists in thymus mitochondria to attempt its purification. Using procedures previously used to purify UCP 1 from BAT mitochondria (Lin & Klingenberg, 1980), we successfully purified UCP 1 from rat thymus mitochondria. The purified protein yielded a single ~33 kDa protein band on a one dimensional SDS-PAGE gel (Chapter 8). Following excision of the protein band and tryptic digestion of the protein, MALDI-TOF and LC-
MS/MS analysis confirmed that the predominant protein was UCP 1 from rat (NDBI accession # P04633) (Chapter 8).

In some rats (but not all), BAT can be seen in the vicinity of the thymus. This begs the question as to whether the UCP 1 we have detected in thymus is actually due to BAT contamination of the thymus (mitochondrial) preparation. There are several reasons why this could not be the case. Firstly, the thymus (brilliant white in colour) is clearly distinguishable from BAT (creamy brown in colour) in colour and shape. Any visible BAT surrounding the thymus was clearly visible and was carefully excised from the thymus. Secondly, the thymocyte preparation employed in this study yields lymphocytes predominantly. In addition, any fat cells (adipocytes) released during the isolation of thymocytes float to the top of the RPMI-1640 medium and are discarded (see Figure 2.3; Chapter 2). Hence minimal WAT and no BAT cells contaminate the thymocyte preparation. In our binding studies, we observe $B_{\text{MAX}}$ values for thymus mitochondria, three quarters those of BAT mitochondria. One would have to envisage that three quarters of the mitochondria in the thymus/thymocyte mitochondrial preparation was due to contamination by BAT mitochondria which is absurd. Furthermore, the rates of oxygen consumption due to proton leak in non-Dowex treated thymus mitochondria from room temperature animals were approximately a third that of non-Dowex treated BAT mitochondria from room temperature animals. Again, one would have to envisage that at least a third of the mitochondria in the thymus mitochondrial preparation can from BAT, which is very unlikely. Our ability to isolate and purify UCP 1 protein from thymus mitochondria (Chapter 8) shows there are significant amounts of UCP 1 in thymus. Contamination by BAT would had to have been extensive to be able to isolate and purify UCP 1 protein. Finally, previous studies have shown that UCP 1 purified from BAT mitochondria can be N-terminally sequenced (Aquila et al., 1985). However, when we employed the University of Leeds to N-terminally sequence UCP 1 purified from thymus, they were unsuccessful and advised us that the N-terminus was blocked (results not shown). Clearly, there is a difference in the chemistry of the N-terminus of UCP 1 from BAT when compared with the N-terminus of UCP 1 from thymus. Taken together, our results irrefutably show the presence of a functioning UCP 1 in rat thymus and thymocyte mitochondria.
The discovery of UCP 1 in thymus and thymocyte mitochondria has implications for understanding the bioenergetics, metabolism and immune function of the thymus. It has been shown that the thymus is innervated by adrenergic receptors, like in BAT (Ritter & Crispe, 1992). Thermogenesis in BAT results from the sympathetic stimulation of β-adrenergic receptors on the surface of BAT cells. This, in terms of the bioenergetics, can't but postulate a similar role for UCP 1 in thymus. In addition, natural proton leak activity has been shown to be lower in thymocytes isolated from UCP 2-deficient mice, when compared with wildtype mice. Krauss et al. (2002) estimated that a significant proportion (50%) of natural proton leak in resting thymocytes was dependent on UCP 2. One might also expect UCP 1 to play a role in in situ mitochondrial natural proton leak in thymocytes. Future studies using UCP 1 KO mice will establish whether UCP 1 has a role in adaptive and or natural leak. In the context of thymocyte development, apoptosis is a prolific event in the lifetime of the thymus (Ritter and Crispe, 1992). In vitro, corticosteroids are known to induce apoptosis in T-lymphocytes (Ritter and Crispe, 1992). Furthermore, Buttergeit et al. (1994) have shown that the pro-apoptotic agent, methylprednisolone, inhibits respiration by about 20% in Concanavalin-A (Con-A)-stimulated thymocytes from rats. Interestingly, this pro-apoptotic agent also increased proton leak across the mitochondrial inner membrane by an unknown mechanism (Buttergeit et al., 1994). The increased metabolic flux due to this increased proton leak, maybe as a result of UCP 1. In addition, the thymus is also the site of maturation and activation of T-lymphocytes and where thymocytes are stimulated to convert to non-dedicated T-cells in mammalian bone-marrow (Ritter & Crispe, 1992). As UCP 1 appears to be expressed in the primary producing T-lymphocyte tissue, the thymus, and not in the secondary T-lymphocyte producing tissue, the spleen (Ricquier & Bouillaud, 2000; Nègre-Salvayre et al., 1997; Figure 3.2.6, Chapter 3), one may postulate a role for UCP 1 in the maturation of early T-lymphocytes. Again, UCP 1 KO mice would be useful in determining the developmental role of UCP 1 in thymus.

Another probable role for UCP 1 in thymus mitochondria is in the regulation of superoxide production. Echtay et al. (2003) and Murphy et al. (2003) has shown
that matrix generated superoxide from Complex I probably activates the proton leak activity of UCP's, which in turn reduce the amount of superoxide generated. Previously, Nègre-Salvayre et al. (1997) have shown that H$_2$O$_2$ production was stimulated by the inhibition of BAT mitochondrial respiration through the addition of GDP. The same authors also observed a similar effect of GDP on mitochondria isolated from spleen and thymus. However, Nègre-Salvayre et al. (1997) then speculated, that since spleen and thymus contain no UCP 1, but express UCP 2 gene, UCP 2 must be the regulator of H$_2$O$_2$ production. However, our novel finding that thymus mitochondria do express UCP 1 protein puts UCP 1 in the frame as a regulator of H$_2$O$_2$ production. The observation that thymocytes require molecular oxygen to undergo apoptosis and that increased levels of H$_2$O$_2$ are pro-apoptotic for cell death mediated by mitochondria (Tonomura et al., 2003), again suggests a role for UCP 1 in apoptosis in the thymus.

Clearly, the discovery of UCP 1 in rat thymus mitochondria has uncovered a new avenue for research into thymus bioenergetics, metabolism, development and function.

[9.2] UCP 3 in BAT, skeletal muscle, thymus and spleen

Using the highly sensitive RT-PCR/PCR methodology, we not only confirm that UCP 3 transcript is expressed in rat BAT, but we go on to show the novel finding that UCP 3 mRNA is expressed in human thymus and spleen (Chapter 3). Our novel finding contrasts with several other research groups on the literature (Gong et al., 1997; Richard et al., 1999; Ricquier & Bouillaud, 2000; Jekabsons et al., 2001) who failed to detect UCP 3 transcript in both human thymus and spleen, using Northern blot analysis. As with the detection of UCP 1, this lack of detection of UCP 3 in human thymus and human spleen implies that UCP 3 mRNA is present in low abundance in these tissues. Future work will investigate the expression of UCP 3 mRNA in other tissues using RT-PCR.

We have recently characterized a novel peptide antibody to UCP 3, which is sensitive and discriminatory for UCP 3 over UCP 2, UCP 1 and other mitochondrial
transporters (Cunningham et al., 2003). Using the UCP 3 peptide antibody, we confirm the expression of UCP 3 protein in BAT and skeletal muscle mitochondria (Chapter 5). Our findings also show that UCP 3-containing mitochondria do not show significant specific GDP binding in skeletal muscle mitochondria (Chapter 6) and that purine nucleotides and fatty-acids do not inhibit or activate uncoupling in skeletal muscle mitochondria (Chapter 7). We confirm but are also able to quantify the increased (2.8-fold) UCP 3 expression observed in skeletal muscle mitochondria isolated from fasted rats (48-hours). The increase in UCP 3 expression during starvation, at a time when energy expenditure is decreased, does not support a primary role for UCP 3 in energy dissipation. These findings are more consistent with a role for UCP 3 in the regulation of fatty-acid oxidation/metabolism than in nonshivering thermogenesis. It has been suggested that the starvation-induced increase in UCP 3 protein expression is caused by elevated free fatty-acids and that UCP 3 somehow plays an important role in their metabolism (Weigle et al., 1998). Dulloo et al. (2001) and Harper et al. (2001) have also proposed that the primary function of UCP 3 in BAT and skeletal muscle maybe involved with the regulation of lipids as fuel substrates and/or in controlling the export of fatty-acids out of the mitochondria during fatty-acid oxidation, rather then the mediation of thermogenesis.

However, we also show that UCP 3 expression is increased in skeletal muscle mitochondria isolated from rats acclimated to the cold and decreased in skeletal muscle mitochondria isolated from rats acclimated to 30°C (Chapter 5). These results show that UCP 3 expression is temperature-sensitive and suggest a role for UCP 3 in thermogenesis. However, cold-exposure, like starvation has been shown to correlate nicely with increasing fatty-acids (Simonyan et al., 2001). In addition, hyperthyroidism increases energy expenditure, in part by increasing metabolic flux and by lowering metabolic efficiency. Thyroid status is therefore a major determinant of basal metabolism and skeletal muscle is a major contributor to basal metabolism in mammals (Blaxter, 1999). In this context, we show that UCP 3 expression is increased in skeletal muscle mitochondria isolated from hyperthyroid rats (Chapter 5). It has also been reported that the hypothyroidism-hyperthyroidism transition is accompanied by an increase in the endogenous levels of mitochondrial
free fatty-acids (Lombardi et al., 2002). Taken together, these observations suggest a role for UCP 3 in fatty acid metabolism.

Using our peptide antibody selective for UCP 3, our study goes onto show the novel findings that UCP 3 protein is present in mitochondria isolated from rat thymus, rat thymocytes and rat spleen (Chapter 5). Cellular fractionation of the spleen shows that UCP 3 is located in mitochondria isolated from reticulocytes, monocytes and lymphocytes. Furthermore, we show the novel findings that starvation increases UCP 3 protein expression in thymus and thymocyte mitochondria and in spleen lymphocyte mitochondria. We also go onto show that UCP 3 does not bind purine nucleotides in spleen mitochondria, as was observed for skeletal muscle mitochondria (Chapter 6). Taken together, these results suggest that UCP 3 may function differently to UCP 1 in BAT and thymus mitochondria.

As has been discussed for skeletal muscle, the increase in UCP 3 expression upon fasting in thymus, thymocyte and spleen lymphocyte mitochondria coincides with increasing levels of free fatty-acids and therefore may suggest a role for UCP 3 in fatty-acid metabolism and/or fatty-acid oxidation in thymus, thymocyte and spleen lymphocyte mitochondria. The fact that UCP 3 is detected in the primary lymphocytes producing tissue (the thymus) and the secondary lymphocyte producing tissue (the spleen) suggests a persistent role for UCP 3 in the developing T-lymphocyte. Interestingly, starvation had no effect on UCP 3 protein expression in spleen mitochondria. This may suggest that UCP 3 functions differently in spleen mitochondria compared to its role in skeletal muscle and thymus/thymocyte mitochondria.

In parallel with UCP 1 and UCP 2, UCP 3 might also play a role in controlling ROS production in thymus and spleen mitochondria. It has previously been shown that the absence of UCP 3 led to an increase in ROS production, thereby implying a role for UCP 3 in preventing the formation of ROS in skeletal muscle (Vidal-Puig et al., 2000). Similarly, like UCP 1 in thymus, UCP 3 may play a central role in apoptosis and thymus development.

Finally, lymphoid atrophy is a well-recognized consequence of nutritional deprivation (Howard et al., 1999). The disproportionate loss of lymphoid tissue with starvation (48-hour) is more pronounced in the thymus than in the spleen (Howard et
al., 1999), and our study has confirmed this (Chapter 5). The increase in UCP 3 expression upon starvation in mitochondria isolated from lymphocyte-rich tissues (i.e. thymus/thymocyte and spleen lymphocytes) may suggest a role for UCP 3 in lymphoid atrophy.

Our novel findings that show UCP 3’s association with immune cells/tissues has revealed a novel bioenergetic route for further investigation into immune cell bioenergetics, metabolism and function.
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Selective detection of UCP 3 expression in skeletal muscle: effect of thyroid status and temperature acclimation


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Abstract

A novel peptide antibody to UCP 3 is characterized which is sensitive and discriminatory for UCP 3 over UCP 2, UCP 1 and other mitochondrial transporters. The peptide antibody detects UCP 3 expression in E. coli, COS cells and yeast expression systems. The peptide antibody detects a single ~33 kDa protein band in mitochondria from isolated rat skeletal muscle, mouse and rat brown adipose tissue, and in whole muscle groups (soleus and extensor digitorum longus) from mice. No 33 kDa band is detectable in isolated mitochondria from liver, heart, brain, kidney and lungs of rats, or gastrocnemius mitochondria from UCP 3 knock-out mice. From our data, we conclude that the peptide antibody is detecting UCP 3 in skeletal muscle, skeletal muscle mitochondria and brown adipose tissue mitochondria. It is also noteworthy that the peptide antibody can detect human, mouse and rat forms of UCP 3. Using the UCP 3 peptide antibody, we confirm and quantify the increased (2.8-fold) UCP 3 expression observed in skeletal muscle mitochondria isolated from 48-h-starved rats. We show that UCP 3 expression is increased (1.6-fold) in skeletal muscle of rats acclimated over 8 weeks to 8 °C and that UCP 3 expression is decreased (1.4-fold) in rats acclimated to 30 °C. Furthermore, UCP 3 expression is increased (2.3-fold) in skeletal muscle from hyperthyroid rats compared to euthyroid controls. In addition, we show that UCP 3 expression is only coincident with the mitochondrial fraction of skeletal muscle homogenates and not peroxisomal, nuclear or cytosolic and microsomal fractions.

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Keywords: Uncoupling protein; UCP 3; Peroxisome; Mitochondria; Antibody; Thyroid; Skeletal muscle

1. Introduction

The function of the novel uncoupling proteins UCP 2 and UCP 3 is still a matter of contention. It has been reported that UCP 2 and UCP 3 act as uncoupling proteins, in the same manner as UCP 1, being conduits for proton translocation across the mitochondrial inner membrane [1]. Other data suggest that UCP 2 and UCP 3, like UCP 1, flip fatty acid anions across the mitochondrial inner membrane thus completing a cycle of protonated fatty acid diffusion and unprotonated fatty acid flipping leading to uncoupling [2]. In contrast, other data from isolated mitochondrial studies show no correlation between increased expression of UCP 2 and UCP 3 and increases in mitochondrial proton leak [3]. Dulloo et al. [4] have demonstrated a correlation between levels of UCP 3 transcript and fatty acid oxidation. Other evidence suggests that UCP 1, UCP 2 and UCP 3 facilitate superoxide translocation across the mitochondrial inner membrane [5]. UCP 2 knock-out mice have no obvious whole body metabolic phenotype to distinguish them from wild-type mice. However, UCP 2 knock-out mice do have an increased resistance to infection by Toxoplasma gondii. This resistance to infection has been attributed to increased reactive oxygen species (ROS) production by mitochondria in their macrophages [6]. Similarly, UCP 3 knock-out mice have no obvious phenotype to distinguish them from wild-

Abbreviations: ATCC, American Tissue Culture Center; BSA, bovine serum albumin; COIII, cytochrome oxidase subunit 3; cyt c, cytochrome c; ECL, enhanced chemiluminescence; EDL, extensor digitorum longus; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethoxy ether) N,N,N',N'-tetraacetic acid; OD, optical density; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SDS, sodium dodecylsulfate; STE, sucrose Tris EGTA buffer; UCP, uncoupling protein

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type mice [7,8]. However, decreased proton leak has been observed in isolated skeletal muscle mitochondria from UCP 3 knock-out mice [8]. By contrast, overexpression of UCP 3 protein in skeletal muscle of mice causes hyperphagia and increases whole body metabolic rate and proton leak of mitochondria in skeletal muscle [9]. However, it is not clear whether the increased leak is attributable to an increased amount of protein in the inner membrane or whether it is a specific function of the UCP 3 protein.

Functional analysis aside, it is possible to get clues to the function of UCPs, and the context in which that function operates, by observing their pattern of expression under various physiological and pathological conditions. Detection of UCP transcript is useful but is not necessarily representative of expression of the functional entity, i.e. the protein. This has clearly been the case for UCP 2 where transcript studies suggested ubiquitous expression in many mammalian tissues [10], whereas protein detection studies suggest UCP 2 is confined to specific tissues, namely lung, spleen, kidney, stomach and white adipose tissue [11]. Few studies have focused on UCP 3 protein expression despite the fact that commercial peptide antibodies to UCP 3 are available from Calbiochem, Chemicon International and Lilly. Peptide antibodies to UCP 3 have also been developed in-house by a number of groups [7,12]. However, in most cases, the sensitivity and discriminatory nature of those antibodies to other UCPs, and mitochondrial transporters in general, have not been satisfactorily demonstrated [12].

Northern blot analysis and Western blot analysis using peptide antibodies to UCP 3, have shown that UCP 3 is found predominantly in skeletal muscle of mammals [10,13,14]. Furthermore, starvation has been shown to increase UCP 3 mRNA levels in mouse skeletal muscle [13] and UCP 3 protein expression in mitochondria from skeletal muscle of rats [3]. Short-term cold exposure also increases UCP 3 protein expression in skeletal muscle mitochondria [15]. UCP 3 mRNA has also been detected in brown adipose tissue [10].

In this study, a novel peptide antibody to UCP 3 is described that is sensitive and discriminatory for UCP 3 over UCP 2, UCP 1 and other mitochondrial transporters. The antibody detects UCP 3 in skeletal muscle and brown adipose tissue as predicted from UCP 3 transcript studies. Using the UCP 3 peptide antibody, we confirm and quantify the increased UCP 3 expression in skeletal muscle mitochondria isolated from starved rats. We show that this specific antibody is sensitive enough for use on whole muscle homogenates. We show that UCP 3 expression is increased in skeletal muscle of rats after long-term cold exposure and that UCP 3 expression is decreased in rats acclimated to 30 °C. We demonstrate that UCP 3 expression is increased in skeletal muscle mitochondria of hyperthyroid rats. Our data also show that UCP 3 expression is coincident with the mitochondrial fraction of skeletal muscle homogenates.

2. Materials and methods

2.1. Isolation of mitochondria from liver, kidney, lungs, brain, brown adipose tissue

Mitochondria were prepared essentially by the method of Chappell and Hansford [16]. Tissues were removed, weighed and placed in ice-cold sucrose Tris EGTA buffer (STE) buffer (250 mM Sucrose, 5 mM Tris—HCl, 2 mM ethylene glycol-bis-(γ-aminooethyl ether) N,N,N',N'-tetraacetic acid (EGTA), pH 7.4). Tissues were chopped finely using scissors and washed several times with STE buffer. The tissue was homogenised by hand using a Dounce homogeniser as follows: four passes with a pestle of 0.26 inch clearance followed by six passes with a pestle of 0.12 inch clearance. The homogenate was centrifuged at 800 × g for 3 min at 4 °C, pelleting blood and debris. The pellet was discarded and the supernatant was centrifuged at 12,000 × g for 10 min at 4 °C yielding a “mitochondrial” pellet. The mitochondrial pellet was resuspended in STE buffer and re-centrifuged as described above. The resulting mitochondrial pellet was resuspended in STE buffer to the desired concentration.

2.2. Isolation of heart and skeletal muscle mitochondria

Skeletal muscle mitochondria from rat hind leg muscle and rat heart mitochondria were isolated by the method of Bhattacharya et al. [17]. Hindquarter muscle (all leg and a small amount of back muscle) and hearts were removed, weighed and placed in ice-cold isolation medium (100 mM sucrose, 9 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Tris—HCl, 46 mM KCl, pH 7.4). Muscle and heart were finely chopped on a pre-cooled glass tile with a razor blade, added to isolation medium (200 ml) containing 0.5% (w/v) bovine serum albumin (BSA) and 0.02% (w/v) nase (Sigma, Protease VII) and incubated on ice, with stirring, for 5 min. The tissue was further disrupted using a Waring commercial blender at half-maximum speed for 15 min. The homogenate was centrifuged at 1500 × g for 3 min at 4 °C. The whitslip lipid layer on top of the supernatant was removed using a spatula and the supernatant was centrifuged at 12,000 × g for 10 min at 4 °C. The mitochondrial pellet was resuspended in ice-cold isolation medium and centrifuged as above. The final mitochondrial pellet was resuspended in ice-cold isolation medium to the desired concentration.

2.3. Preparation of muscle groups for analysis

The hind limb muscles soleus and extensor digitorum longus (EDL) were removed, ensuring complete removal of associated tendons and connective tissue and placed in ice-cold muscle isolation medium (100 mM sucrose, 9 mM EDTA, 1 mM EGTA, 46 mM KCl, 100 mM Tris—HCl, pH 7.5). Muscles were finely chopped on a pre-cooled glass tile.
and frozen in approximately 10 ml of liquid nitrogen in a porcelain mortar. A porcelain pestle was used to grind the frozen muscle to a fine powder. Once ground, EDL and muscles from each animal were individually resuspended in 500 μl of cold muscle isolation medium. Samples were aliquoted in 100 μl volumes and stored at —20 °C.

2.4. Subcellular fractionation of muscle and liver tissue

Fractionation of skeletal muscle was performed essentially as described for fractionation of liver by Hartl et al. [18] with minor modifications from Gouveia et al. [19] using minced muscle or liver from 48-h-starved Wistar rats.

2.5. Expression of protein and isolation of inclusion bodies from E. coli

A colony of E. coli cells, BL21 pLysS, which had been transfected with the appropriate plasmid (pET 3a-haUCP 1, pET 21d-hUCP 2 or pET 21d-hUCP 3) containing cDNA for the UCP required was inoculated into 5 ml of LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, 100 mg ampicillin in 1 l). The colony was amplified overnight and the following day, the culture was diluted to 500 ml and grown until OD600 had reached 0.4. Induction of expression of the UCP was carried out with 1 mM isopropyl β-d-thiogalactopyranoside (IPTG). Two hours following the addition of IPTG, the cells were harvested at 10,000 x g and the bacterial pellets frozen at —20 °C for at least an hour. Pellets were then thawed and inclusion bodies were isolated as described in Echtay et al. [20].

2.6. Expression of UCP 3 in yeast

The human UCP 3 yeast expression vector pYU3HSL was a gift from Dr. Daniel Ricquier (Centre de Recherche sur l’Endocrinologie Moleculaire et le Developpement, Meudon, France). The UCP 3 transcript was excised from pYU3HSL and subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen). COS-1 cells were obtained from the American Tissue Culture Center (ATCC). All cell culture reagents were supplied by Gibco (Invitrogen). COS-1 cells were seeded in six-well plates at ~ 2 x 10^5 cells per well and grown overnight at 37 °C in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10 U/ml penicillin, 100 mg/ml streptomycin sulfate and 10% foetal calf serum under 95% air/5% CO₂, then transiently transfected with pcDNA3.1 containing the UCP 3 transcript and pcDNA3.1 alone using Fugene (Roche) according to the manufacturer’s instructions. Twenty-four hours following transfection 100 μl of sodium dodecylsulfate (SDS) sample buffer (0.0625 M Tris–HCl, pH 6.8, 10% (w/v) glycerol, 4% (w/v) SDS, 5% (w/v) β-mercaptoethanol and 0.005% (w/v) bromophenol blue) was added to the wells and the cells were homogenised by being drawn up repeatedly through a narrow bore needle. The cell homogenates were stored at —20 °C until analysis.

COS-1 cells were also transfected with pcDNA3.1 containing the UCP 3 transcript and stable clones were isolated using neomycin selection. Colonies were isolated when they had grown to ~ 2 mm in diameter and expanded in selection media. Stable clones were harvested in SDS sample buffer as described above. Stable clones with the pcDNA3.1 vector alone were also isolated for use as a negative control.

2.8. Protein determination

Tissue and mitochondrial protein concentration was determined according to the procedure described by Markwell et al. [22].

2.9. Preparation of samples for polyacrylamide gel electrophoresis

Samples (10–100 μg protein) were solubilised in sample buffer; 0.0625 M Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 1.66% β-mercaptoethanol (added directly prior to use) and 0.001% bromophenol blue. Prior to loading on gel, samples were vortexed and pulsed in a minifuge before boiling at 100 °C for 5 min.

2.10. Polyacrylamide gel electrophoresis

Gels were run using the Laemmli [23] system for denaturing gels. Slight modifications were used as de-
scribed: 0.375 M Tris–HCl, pH 8.7 and 0.1% SDS were used in all solutions, except for the gels for the subcellular fractionation and peroxisome samples, which had 0.75 M Tris. Linear 12% polyacrylamide gels were prepared from a stock solution containing 30% (w/v) acrylamide; 0.4% (w/v) bis-acrylamide. Samples were prepared using Laemmli sample buffer containing either 5% (w/v) β-mercaptoethanol or 20–100 mM dithiothreitol, and subsequently incubated for 5 min at 100 °C or 20 min at 70 °C. The gel dimensions, for the subcellular fractionation and peroxisome samples, were 14 cm x 10 cm x 1 mm. Gels for whole muscle samples were 9 cm x 7 cm x 1.5 mm. All other gels were 9 cm x 7 cm x 0.75 mm. All gels were run on a Protean II or Protean III (Bio-Rad) gel system.

2.11. Western blotting analysis

Following polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose or polyvinylidene difluoride (PVDF) (Millipore). Transfer was achieved using a semi-dry apparatus for 2 h at 110 mA or a Bio-Rad Trans-Blot Transfer Cell for 1–4 h at 20 V/cm. After transfer was complete, blots were blocked for 1 h in phosphate-buffered saline (PBS)-Tween (0.14 M NaCl, 2.7 mM KCl, 11.5 mM Na_2PO_4, 1.8 mM KH_2PO_4, pH 7.4 with 0.1% Tween-20) containing 5% non-fat dried milk (Marvel). This was followed by three 10-min washes with PBS-Tween.

Blots were probed with primary antibody overnight at 4 °C in the same solution containing 1:5000 dilution of an affinity-purified anti-UCP 3 peptide antibody, a 1:5000 dilution of an affinity-purified anti-UCP 2 peptide antibody, a 1:5000 dilution of an affinity-purified anti-UCP 1 peptide antibody, a 1:1000 dilution of rabbit antisera directed to human Pex14p [24], a 1:5000 dilution of a monoclonal antibody directed to the α-subunit of the mitochondrial ATP synthase (clone 7H10-BD4, Molecular Probes), a 1:500 dilution of purified mouse anti-cytochrome c monoclonal antibody (BD PharMingen), a 1:1000 dilution of peptide antibody to subunit III of cytochrome oxidase or a 1:10,000 dilution of the full-length UCP 1 antibody. These steps were followed by three 10-min washes with PBS-Tween.

Blots were then probed with secondary antibody incubated in 1:10,000 dilution of a goat anti-mouse IgG horse-radish peroxidase (HRP) conjugate (for cytochrome c), a donkey anti-sheep IgG HRP conjugate (for full-length UCP 1 antibody) or a goat/donkey anti-rabbit IgG HRP conjugate (for all others) secondary antibody in PBS-Tween containing 5% non-fat dried milk for 1 h. After a further three 10-min washes in PBS-Tween, the blots were developed using an enhanced chemiluminescence (ECL) detection system (Amersham-Biosciences) and visualized by exposure to X-ray film. The ATP synthase (F_oF_1) and Pex14p blots were developed using chloronaphthol (Sigma) and hydrogen peroxide as substrates for the HRP-linked secondary antibody. The antibody to the F_1β was raised by the β-subunit of the ATP-synthase from Neurospora crassa.

2.12. Densitometry

Following Western blot development, the relative abundance of UCP 3 was determined by using densitometry. The band intensities of the exposed film were analysed using Scion Imaging Software (Scion Corporation, Maryland, USA).

2.13. UCP antibodies

Peptide synthesis, conjugation of peptides to BSA and injection of rabbits was undertaken by Eurogentec (Parc Scientifique du Sart Tilman, 4102 Seraing, Belgium). Polyclonal antibodies in the resulting rabbit anti-sera were affinity-purified on a protein A column. The peptides to which polyclonal antibodies were raised are as follows: UCP 1, 145...CHLHGIKPRYGNA...158; UCP 2, 147...QARAGGGRYQSTVEA...162; UCP 3, 141...TGGERKRYGTDAYRC...156. The full-length antibody to UCP 1 was a gift from Daniel Ricquier (Centre de Recherche sur l’Endocrinologie Moléculaire et la Développement).

3. Results

In a bid to distinguish between the uncoupling proteins UCP 1, UCP 2 and UCP 3, antibodies were raised to peptide sequences unique to each uncoupling protein based on similarities with the rat sequences. The resulting affinity-purified polyclonal antibodies were tested for their ability to detect UCP 1, 2 and 3 protein expressed by E. coli (Fig. 1). Blot A shows that anti-UCP 3 peptide antibodies detected only UCP 3 (lane 1) and not UCP 2 (lane 2) or UCP 1 (lane 3). Blot B demonstrates that UCP 2 antibodies could not discriminate between the UCPs and detected UCP 3 (lane 1), UCP 2 (lane 2) and UCP 1 (lane 3). Blot C shows that the UCP 1 antibodies only detected UCP 1 (lane 3) and not UCP 2 (lane 2) or UCP 3 (lane 1). One general observation is that all expressed uncoupling proteins had apparent

![Fig. 1. Anti-UCP 3 peptide antibody is specific for E. coli expressed UCP 3. Lane 1, 5 μg of hUCP 3; lane 2, 20 μg hUCP 2; lane 3, 20 μg haUCP 1. Polyclonal peptide antibodies to rUCP 3 (Blot A), rUCP 2 (Blot B) and rUCP 1 (Blot C) were used at a dilution of 1:5000. Exposure time was the same for all samples (30 s).](image-url)
molecular masses circa 32–34 kDa. All blots were exposed for equal time periods (30 s).

For the purpose of estimating increases in UCP 3 protein expression, we were able to show that there is a linear relationship between the amount of E. coli expressed UCP 3 and the resultant intensity of the bands on the exposed film of a given immunoblot (Fig. 2).

In addition to detection of UCP 3 expressed in E. coli, the polyclonal peptide antibodies to UCP 3 were able to detect UCP 3 transiently expressed in mammalian COS cells (Fig. 3A) using 10^6 (lane 1) and 10^7 (lane 2) cells. No protein was detected in COS cells transfected with an empty vector (lane 3). As might be expected, the polyclonal peptide antibodies to UCP 1 could not detect anything in mammalian COS cells transiently expressing UCP 3 at either cell concentration (results not shown). Fig. 3B shows that the polyclonal peptide antibodies to UCP 3 were able to detect UCP 3 expressed in a stably transfected clone of mammalian COS cells without having to isolate mitochondria (lane 1). Anti-UCP 2 or anti-UCP 1 peptide antibodies did not detect this band (results not shown). No protein was detected in control cells (lane 2).

UCP 3 was also expressed in yeast cells (Fig. 3C). The polyclonal peptide antibodies to UCP 3, detected a ~ 33 kDa band in cell homogenate containing plasmid expressing UCP 3 (lane 1) but detected nothing in yeast control cell homogenate (lane 2).

Fig. 4A shows that our anti-UCP 3 antibody detected a 33 kDa band in yeast cells expressing UCP 3 (lane 1), in mitochondria isolated from skeletal muscle of non-starved rats (lane 4) (no particular muscle group selected), in mitochondria isolated from skeletal muscle of starved rats (lane 5) and in mitochondria from brown adipose tissue of cold-adapted rats (lane 7). No UCP 3 was detected in rat liver mitochondria (lane 2), rat heart mitochondria (lane 3), mitochondria from rat brain (lane 6), mitochondria from rat kidney (lane 8) or mitochondria from rat lung (lane 9).

Although 100 µg of mitochondria were used in the blots in Fig. 4, the anti-UCP 3 peptide antibody will detect UCP 3 at total skeletal muscle mitochondrial protein concentrations of
Fig. 5. Anti-UCP 3 peptide antibody detects UCP 3 in gastrocnemius mitochondria from wild-type but not UCP 3 knock-out mice. Immunoblots of UCP 3 peptide antibody (Blot A) and antibody to the β-subunit of the F$_1$-ATP synthase (Blot B): lane 1, yeast (~50 μg) expressing UCP 3; lane 2, gastrocnemius muscle mitochondria from C57BL/6J wild-type (WT) 6-week-old female mice; lane 3, gastrocnemius muscle mitochondria from C57BL/6J UCP 3 knock-out 6-week-old female mice; lane 4, rat liver mitochondria (100 μg).

$32.5\text{kDa}$

Fig. 6. Anti-UCP 3 peptide antibody detects equivalent amounts of UCP 3 in brown adipose tissue mitochondria from wild-type and UCP 1 knock-out mice. Immunoblots of UCP 3 peptide antibody (Blot A) and antibody to the β-subunit of the F$_1$-ATP synthase (Blot B): lane 1, yeast (~50 μg) expressing UCP 3; lane 2, brown adipose tissue mitochondria from C57BL/6J wild-type (WT) mice (100 μg); lane 3, brown adipose tissue mitochondria from C57BL/6J UCP 1 knock-out mice (100 μg); lane 4, mouse liver mitochondria (100 μg). The relative abundance of UCP 3 in brown adipose tissue mitochondria from UCP 1 wild-type (sample 1) and UCP 1 knock-out mice (sample 2) is given in panel C (mean ± S.E.M.). (NS, not significant).

$32.5\text{kDa}$

$F_1\beta$

Fig. 7. The effect of temperature acclimation of rats on UCP 3 expression in skeletal muscle mitochondria. (A) UCP 3 peptide antibody and (B) antibody to cytochrome oxidase subunit III to: lane 1, yeast expressing UCP 3 (~50 μg); lane 2, skeletal muscle mitochondria from rats kept at 20 °C (100 μg); lane 3, skeletal muscle mitochondria (100 μg) from rats exposed to 30 ± 2 °C for 8 weeks; lane 4, skeletal muscle mitochondria from rats exposed to the cold (8 ± 2 °C) for 8 weeks (100 μg); lane 5, liver mitochondria (100 μg) from rats at 20 ± 2 °C for 8 weeks. (C) The ratio of UCP 3 protein expression to that of cytochrome oxidase, subunit 3 (COIII), for (sample 1) skeletal muscle mitochondria from rats acclimated to 20 °C, (sample 2) skeletal muscle mitochondria from rats acclimated to 30 °C and (sample 3) skeletal muscle mitochondria from rats acclimated to 8 °C. Data are from three separate preparations. *$P<0.0047$ **$P<0.0113$.

The primary in vivo site of UCP 3 mRNA and protein expression is skeletal muscle. In Fig. 5, the peptide antibody to UCP 3 detects a 33 kDa protein in the yeast expressing UCP 3 (lane 1) and in mitochondria isolated from the gastrocnemius of wild-type mice (lane 2). However, no 33 kDa protein is detected in mitochondria isolated from the gastrocnemius of UCP 3 knock-out mice (lane 3) or in rat liver mitochondria (lane 4).

The most abundant UCP in brown adipose tissue would be UCP 1 [10]. Therefore, we wished to reassure ourselves that our UCP 3 antibody was not detecting native UCP 1. Fig. 6A and B demonstrate that the intensity of UCP 3 detection is not different when comparing brown adipose mitochondria from wild-type (lane 2) and UCP 1 knock-out mice (lane 3) and this is demonstrated graphically (Fig. 6C). As expected, UCP 3 was detected in yeast expressing UCP 3 (lane 1) but not in mouse liver mitochondria (lane 4).

The immunoblot in Fig. 7A shows that expression of UCP 3 protein in rat skeletal muscle is sensitive to temperature acclimation of the animal. As previously demonstrated, UCP 3 is detectable using the polyclonal anti-UCP 3 peptide antibody in yeast cells expressing UCP 3 (lane 1) and in mitochondria from non-starved rats housed at a constant 20 °C room temperature (lane 2). Skeletal muscle
mitochondria isolated from rats acclimated to a temperature of 30 °C demonstrates a 3-fold decreased UCP 3 expression (lane 3) compared to those at 20 °C, while skeletal muscle mitochondria isolated from rats acclimated to a temperature of 8 °C for 8 weeks (lane 4) demonstrate increased UCP 3 expression compared to those at 20 °C (lane 2). As expected, no UCP 3 was detected in liver mitochondria (lane 5). The temperature acclimation experiments were repeated several times with similar results. Fig. 7B gives a relative measure of the amount of mitochondrial protein loaded in each lane and demonstrates that the amount of mitochondrial protein loaded in each lane cannot account for the differences observed in the amount of UCP 3 protein in lanes 1-4. In addition, there is a significant (P < 0.0047; n = 3) 1.4-fold decrease in UCP 3 expression observed in mitochondria isolated from skeletal muscle of rats acclimated to 30 °C when compared to mitochondria from rats acclimated to 20 °C and there is a significant (P < 0.0113; n = 3) 1.6-fold increase in UCP 3 expression observed in mitochondria isolated from skeletal muscle of rats acclimated to 8 °C when compared to mitochondria from rats acclimated to 20 °C (Fig. 7C).

Data in the literature show that thyroid hormone administration increases transcripts of UCP 3 in skeletal muscle of mice and rats [10]. Fig. 8A shows a representative set of samples where UCP 3 protein expression is increased in skeletal muscle mitochondria from hyperthyroid rats (lane 5) compared to euthyroid controls (lane 3), an increase comparable with that found in mitochondria from starved animals (lane 4). The peptide antibody to rat UCP 3 detects a 33 kDa band in liver mitochondria (lane 2) but detects UCP 3 expressed in yeast (lane 1) as expected. Fig. 8B shows a representative set of samples indicating that differences in UCP 3 expression (Fig. 8A) cannot be accounted for by differences in lane loading as indicated by cytochrome oxidase subunit 3 (COIII) detection (Fig. 8B). Fig. 8C gives the ratio of UCP 3 expression to COIII expression for four separate preparations determined in triplicate. The results show a 2.3-fold (P < 0.002, n = 4) increase in UCP 3 expression in the hyperthyroid state with respect to mitochondria from euthyroid animals. In addition, Fig. 8C shows a 2.8-fold (P < 0.001, n = 4) increase in UCP 3 expression in mitochondria from 48-h-starved animals with respect to mitochondria from euthyroid control rats.

The amino acid motif used to define mitochondrial (anion) transporters has also been found to occur in transporters of the peroxisomal membrane [25]. Thus it seemed possible that UCP 3 might also exist in peroxisomes. A subcellular fractionation of skeletal muscle from 48-h-starved rats was performed in order to separate peroxisomes (identified using anti-Pex14p) from mitochondria (identified using antibody to the anti-Fja subunit of the ATP synthase) (Fig. 9). Pure rat liver peroxisomes (lanes 1 and 14) were also identified using the anti-Pex14p antibody (blot B) and despite a small amount of mitochondrial contamination (blot C), it can be seen that the UCP 3 antibody does not react with pure peroxisomes from rat liver (blot A). The UCP 3 antibody (blot A) detects protein in the supernatant from the 600 x g centrifugation of rat skeletal muscle homogenate (lanes 2 and 13), which is coincident with mitochondrial protein detection (blot C). However, peroxisomes are not detected in the 600 x g supernatant fraction from rat skeletal muscle homogenate (blot B). The pellet from the 600 x g centrifugation (cells and nuclei) (lanes 3 and 12) contains small amounts of UCP 3 (blot A), no peroxisomes (blot B) and some mitochondria (blot C). The resulting centrifugation of the 600 x g supernatant at 2350 x g resulted in a pellet termed heavy mitochondria (lanes 4 and 11) which contained a strong band for UCP 3 (blot A), a strong band for mitochondria (blot C) but no detection of peroxisomes (blot B). The supernatant from the centrifugation at 2350 x g was then centrifuged at 12,300 x g yielding a supernatant termed cytosolic and microsomal protein (lanes 5 and 10). A small amount of UCP 3 (blot A) and mitochondria (blot C) but no peroxisomes (blot B) were found in this fraction. The pellet from the 12,300 x g centrifugation contains a fraction termed light mitochondria (lanes 6 and 9). This fraction contains a lot of UCP 3 (blot
A), a lot of peroxisomes (blot B) and a lot of mitochondria (blot C). Attempts to purify peroxisomes from this fraction using discontinuous Nycodenz density gradient centrifugation were not very successful. As shown in Fig. 9, no major enrichment of Pex14p can be observed in the organelles obtained after this procedure (compare lanes 8 and 9, panel B). However, a significant depletion (ca. 50%) of both F_1\alpha and UCP 3 in these organelles can be easily observed (compare lanes 8 and 9 of panels A and C). Taken together, these data suggest (indicate) that UCP 3 (like F_1\alpha) is a mitochondrial protein (or is not a peroxisomal protein). An equivalent tissue fractionation was performed on rat liver from non-starved rats but no UCP 3 was detectable in any fractionation (results not shown).

Fig. 10A demonstrates that the anti-UCP 3 antibody can detect UCP 3 in whole muscle homogenates without the need to isolate mitochondria. Lane 1 demonstrates the anti-UCP 3 antibody detecting UCP 3 in yeast cells expressing hUCP 3. UCP 3 could be detected in mouse EDL muscle (lanes 2 and 3) a predominantly glycolytic muscle and in mouse soleus muscle (lanes 4 and 5) a predominantly oxidative muscle. In Fig. 8B, antibodies to cytochrome c were used to give a relative measure of mitochondria density in the muscle groups (lanes 2–5). This particular antibody to cytochrome c works well on mammalian tissue but is known not to detect cytochrome c in yeast, as exemplified in lane 1.

Interestingly, the antibody to full-length UCP 1 detected a 33 kDa protein in cells transiently transfected with plasmid containing hUCP 3 and rat skeletal muscle mitochondria (results not shown).

4. Discussion

Although the function of UCP 3 is still contentious, clues to its physiological function can be obtained from observing their pattern of expression. As the protein is the functional entity, sensitive and discriminatory UCP detection antibodies are desirable. Northern blot analysis has shown that UCP 3 is expressed predominantly in skeletal muscle but also in brown adipose tissue [13]. However, limited immunodetection data exists for UCP 3. In this study, we demonstrate the selectivity and sensitivity of a peptide antibody to UCP 3.

Using uncoupling proteins purified from inclusion bodies expressed in E. coli, we showed that the anti-UCP 3 peptide antibody is selective for UCP 3 over UCP 2 and UCP 1 (Fig. 1) and that intensity of the UCP 3 bands correlates with abundance of UCP 3 protein (Fig. 2). In addition, the anti-UCP 1 antibody would also appear to be selective, detecting only E. coli expressed UCP 1 over UCP 2 and UCP 3 (Fig. 1).

The anti-UCP 3 peptide antibodies were also capable of detecting UCP 3 expressed in COS and yeast cells (Fig. 3). The same peptide antibody detected nothing in COS cells or yeast cells transfected with empty vector although both cell types contain mitochondria which themselves express transporters of an equivalent size. As might be
predicted, antibodies to the UCP 1 peptide antibody were unable to detect UCP 3 in these cells. But interestingly, antibodies to full-length UCP 1 were able to detect UCP 3 expressed in COS cells and in rat skeletal muscle mitochondria.

The pattern of the UCP 3 protein detection data (Fig. 4) are consistent with the UCP 3 mRNA detection data [10]. The antibody detected UCP 3 protein in skeletal muscle mitochondria and brown adipose tissue of rats. No UCP 3 was detected in mitochondria from tissues known to contain UCP 2 protein (kidney, brain and lungs) [11,26], nor was UCP 3 protein detected in heart or liver. The conclusion from these data is that the anti-UCP 3 antibody was detecting UCP 3 and not another member of the mitochondrial anion transporter family.

Our polyclonal UCP 3 peptide antibody can detect UCP 3 in mitochondria isolated from gastrocnemius of wild-type but not UCP 3 knock-out mice. Furthermore, in Fig. 6, it can be seen that the UCP 3 antibody detects UCP 3 to the same extent in brown adipose tissue of wild-type and UCP 1 knock-out mice. Taken together, it can be concluded that the anti-UCP 3 peptide antibody is selective for UCP 3 and is not detecting another member of the mitochondrial anion transport family.

Having established the discriminatory nature of the anti-UCP 3 peptide antibodies, we looked at the effect of long-term temperature acclimation on expression of native UCP 3. It was shown that cold-acclimated rats had increased (1.6-fold) UCP 3 expression compared to controls kept at room temperature (Fig. 7), whereas rats acclimated to 30 °C had decreased (1.4-fold) UCP 3. The suggestion is that UCP 3 protein expression is temperature-sensitive. These results again show our antibody to be sensitive to changes in the amount of UCP 3 expression per milligram of mitochondria. These results possibly contrast with those of Boss et al. [13] who showed that UCP 3 mRNA levels in mouse skeletal muscle do not increase with 48 h (short-term) cold exposure yet possibly compliment those of Simonyan et al. [15] who show that UCP 3 protein levels increase after 24 h (short-term) cold exposure.

Thyroid hormone is a major determinant of basal metabolism [27] and there are reports in the literature that thyroid hormone administration increases transcripts of UCP 3 in skeletal muscle of mice and rats [10]. In this study, we found that the hyperthyroid condition increases UCP 3 protein expression 2.3-fold (Fig. 8). The data are consistent with a fatty acid-dependent increase in proton leak in skeletal muscle mitochondria [28]. In addition, we were able to quantify (~2.8-fold) and confirm a previously observed [3] increase in UCP 3 protein expression in skeletal muscle mitochondria from starved rats. No increase in proton leak was observed in that instance [3]. In this context, as previously pointed out by others, one would expect to see an increase in UCP 3 levels upon starvation, as opposed to the observed decrease [3,13]. However, if UCP 3 has a role in fatty acid oxidation as suggested by Dulloo et al. [4], then increased UCP 3 expression on starvation would be consistent with that role.

Recent evidence demonstrated that transporters across the peroxisomal membrane also contain the ‘mitochondrial’ transporter motif [25]. However, subcellular fractionation of skeletal muscle from starved rats demonstrated that UCP 3 was exclusive to mitochondrial fractions (Fig. 9).

The sensitivity of the anti-UCP 3 antibody in detecting UCP 3 is further emphasized by the fact that there is no need to isolate mitochondria from muscle for UCP 3 to be detected. Our anti-UCP 3 antibody was able to detect UCP 3 associated with mitochondria in homogenates of mouse EDL muscle, a predominantly glycolytic muscle and mouse soleus muscle, a predominantly oxidative muscle. Although these particular results aren’t novel, they confirm those of Giacobino [14] and compliment studies on glycolytic and oxidative muscle from humans [12].

In conclusion, our data shows that we have a peptide antibody raised to a rat UCP 3 sequence that is sensitive and discriminatory for UCP 3 over UCP 2, UCP 1 and other mitochondrial transporters. The UCP 3 peptide antibody also detects UCP 3 in isolated mitochondria from skeletal muscle and brown adipose tissue of rats and mice but not mitochondria isolated from UCP 3 knock-out mice. UCP 3 is also detectable using this antibody in whole mouse muscle homogenates without the need for isolation of mitochondria. We confirm and quantify the results of Cadenas et al. [3] that UCP 3 expression is increased in mitochondria isolated from starved animals. We show that UCP 3 expression is increased in cold-acclimated rats and decreased in rats acclimated to 30 °C, suggesting temperature-sensitive expression. In addition, we show that UCP 3 expression is thyroid-sensitive.

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