Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2005/03/23/M413315200.DC1.html

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 15, Issue of April 22, pp. 15534–15543, 2005
© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Identification of a Functioning Mitochondrial Uncoupling Protein 1 in Thymus*[S]

Received for publication, November 26, 2004, and in revised form, January 18, 2005
Published, JBC Papers in Press, January 28, 2005, DOI 10.1074/jbc.M413315200

Audrey M. Carroll‡, Lee R. Haines§, Terry W. Pearson§, Padraic G. Fallon‡, Caitríona M. Walsh‡, Clare M. Brennan‡, Eamon P. Breen‡, and Richard K. Porter‡¶

From the ‡Department of Biochemistry, Trinity College Dublin, Dublin 2, Ireland and the §Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 3P6, Canada

We present evidence that rat and mouse thymi contain mitochondrial uncoupling protein (UCP 1). Reverse transcriptase-PCR detected RNA transcripts for UCP 1 in whole thymus and in thymocytes. Furthermore, using antibodies to UCP 1 the protein was also detected in mitochondria isolated from whole thymus and thymocytes but not in thymus mitochondria from UCP 1 knock-out mice. Evidence for functional UCP 1 in thymus mitochondria was obtained by a comparative analysis with the kinetics of GDP binding in mitochondria from brown adipose tissue. Both tissues showed equivalent B_{max} and K_D values. In addition, a large component of the nonphosphorylating oxygen consumption by thymus mitochondria was inhibited by GDP and subsequently stimulated by addition of nanomolar concentrations of palmitate. UCP 1 was purified from thymus mitochondria by hydroxyapatite chromatography. The isolated protein was identified by peptide mass mapping and tandem mass spectrometry by using MALDI-TOF and LC-MS/MS, respectively. We conclude that the thymus contains a functioning UCP 1 that has the capacity to regulate metabolic flux and production of reactive oxygen-containing molecules in the thymus.

UCP1 (uncoupling protein 1 also known as UCP and thermogenin) has been associated exclusively with brown adipose tissue (BAT)[1] (1, 2) and is a prerequisite for nonshivering thermogenesis in mammals. UCP 1 is known to transport protons and dissipates the proton electrochemical gradient (Δp) across the mitochondrial inner membrane. UCP 1 thus acts as a major regulator of metabolic flux in mitochondria and as a heat regulator in the whole animal (1–4). Recent evidence obtained *in vitro* also suggests that UCP 1 also plays a role in regulating superoxide production by mitochondria (5–8).

UCP 1 synthesis, mitochondrial biogenesis, and thermogenesis are controlled by sympathetic nerve activity and thyroid status. Isolated brown adipocytes from cold-acclimated animals have increased oxygen consumption compared with those from room temperature animals, and the mitochondria isolated from active BAT are uncoupled (1). Although the mechanism of the uncoupling phenomenon is still a matter of investigation (9–11), it is known that long chain fatty acids are required for uncoupling activity and that purine nucleotides inhibit uncoupling through UCP 1 in mitochondria and in the reconstituted systems. The purine nucleotide-binding site on UCP 1 in mitochondria faces the cytosolic side of the inner membrane, and in the presence of saturating amounts of purine nucleotides *in vitro*, oxygen consumption rates due to UCP 1 activity are inhibited. Subsequent addition of nanomolar concentrations of long chain free fatty acids *in vitro* restores the activity of UCP 1-containing mitochondria, and oxygen consumption rate increases (1, 4).

UCP 1 transcripts in BAT have been determined by using Northern blot analysis, and the UCP 1 protein has usually been detected by using polyclonal antibodies specific for the full-length protein. In addition, UCP 1 abundance in BAT (12–14) and the degree of masking (binding sites already occupied) of the purine nucleotide-binding sites (15) have been measured by binding of labeled GDP to mitochondria. To date, UCP 1 protein has not been found in any tissue other than BAT, although it has been sought in liver, heart, epididymal white adipose tissue, parametrial white adipose tissue, and thigh muscle (4, 16); and a previous study, using Northern blot analysis, failed to detect UCP 1 transcripts in thymus (17). However, in this study, we have detected UCP 1 transcripts in whole thymus and thymocytes by using RT-PCR. We have detected UCP 1 protein by using immunoblotting in mitochondria isolated from whole thymus and thymocytes. We have purified and identified UCP 1 from thymus mitochondria by using mass spectrometry. We have also shown that mitochondria isolated from thymus bind GDP with kinetics consistent with the presence of UCP 1 and have a GDP-sensitive and fatty acid-dependent proton leak indicative of the presence of UCP 1.

MATERIALS AND METHODS

Tissue Sources and Isolation of Mitochondria—Wild-type C57BL/6J mice, female CD-1 mice (20–25 g), and female Wistar rats (*Rattus norvegicus*; 180–200 g) were provided by the BioResources Unit, Trinity College Dublin. UCP 1 knock-out mice on a C57BL/6J background (18), originally provided by Dr. Leslie Kozak, were bred in-house. All mice and rats were housed in a specific pathogen-free facility and were fed *ad libitum* unless otherwise stated. Some samples of brown adipose tissue mitochondria from UCP 1 knock-out and wild-type mice, both on a C57BL/6J background, were gifts from Dr. Jan Nedergaard (Werner-

* This work was supported by The Human Frontiers Science Programme Organization Grant RG307/98, Bioresearch Ireland, Science Foundation Ireland, IRCSET, and by a Discovery grant (to T. W. P.) from the Natural Sciences and Engineering Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains a figure.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, Trinity College Dublin, Dublin 2, Ireland. Tel.: 353-1-608-1617; Fax: 353-1-677-2400; E-mail: rkporter@tcd.ie.

[1] The abbreviations used are: BAT, brown adipose tissue; BSA, bovine serum albumin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HTP, hydroxyapatite; LC-MS/MS, liquid chromatography/tandem mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; octyl-POE, octylpentaoctylethylene; RT, reverse transcription; UCP, uncoupling protein; VDAC, voltage-dependent anion channel.

This paper is available on line at http://www.jbc.org

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on September 17, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2005/03/23/M413315200.DC1.html

Gren Institute, Stockholm, Sweden). Skeletal muscle mitochondrial samples from UCP 3 knock-out (19) and wild-type mice, both on a C57BL/6J background, were gifts from Dr. Jean-Paul Giacobino (University of Geneva, Switzerland).

Thymocytes were isolated from Wistar rats (180–200 g) essentially as described by Buttgereit *et al.* (20). The thymus was removed from the rat, trimmed clean of connective tissue and brown fat (if present), and transferred into RPMI 1640 (Invitrogen) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma). A single cell suspension was prepared by passage through 70-μm nylon sieves (Falcon). The thymocyte suspension was washed extensively. The viability of cells was >99% by trypan dye exclusion. Cells were incubated with fluorescein isothiocyanate-conjugated mouse anti-rat Thy-1 (CD90; MRC OX-7; Serotec) or a fluorescein isothiocyanate-conjugated mouse IgG1 isotype control (Pharmingen). Data were collected on a FACScan flow cytometer (BD Biosciences) and analyzed using Cellquest software.

Oxygen consumption rates by thymocytes isolated from rats were measured in the aforementioned medium using an Oxygraph Respirometer (Oroboros™, Innsbruck, Austria). Mitochondria isolated from whole mouse thymus, whole rat thymus, rat thymocytes, rat kidney, and rat and mouse liver were isolated by homogenization followed by differential centrifugation according to the procedure of Chappell and Hansford (21). Mitochondria were isolated from rat skeletal muscle by mincing the muscle and treating the tissue with nagarse (protease VII, Sigma). Mitochondria were separated from the homogenate by differential centrifugation according to the procedure of Bhattacharya *et al.* (22). Mitochondrial protein concentrations were determined by the reduction of Folins-Ciocalteau phosphomolybdic-phosphotungstic reagent according to the method of Markwell *et al.* (23).

PCR and Analysis of the Products—Total RNA was purified from rat BAT, whole rat thymus, rat Thy-1-positive thymocytes, and liver using Tri Reagent™ (Sigma) containing guanidine thiocyanate. Total RNA (1 μg) was reverse-transcribed to cDNA by using avian myeloblastosis virus-reverse transcriptase and random primers (Promega, Southampton, UK). Primers used to amplify UCP 1 were as described by Carroll and Porter (24). Primers for application of the glyceraldehyde-3-phosphate dehydrogenase sequence were used as a housekeeping gene control. Glyceraldehyde-3-phosphate dehydrogenase (GenBank™ accession number M32599) primers were designed according to Tokunga *et al.* (25). PCR amplification conditions were as follows: initial denaturation of 5 min at 95 °C followed by 35 cycles of 45 s at 95 °C, 45 s at 55 °C, and 2 min at 72 °C with a final extension for 10 min at 72 °C. The PCR mixtures were electrophoresed on a 1% (w/v) agarose gel incorporating ethidium bromide and visualized under ultraviolet light.

Polyacrylamide Gels and Immunoblot Analysis—One-dimensional SDS-PAGE under reducing conditions was used to examine UCP 1 purity after hydroxyapatite chromatography. Proteins were detected by staining gels with colloidal Coomassie Brilliant Blue G-250 (26). This stain is very sensitive (can detect 8–10 ng of protein in a single gel band or spot) and is compatible with the post-staining processing required for mass spectrometry (see below).

SDS-PAGE (reducing conditions) was also used to separate proteins prior to immunoblot analysis. UCP 2 purified from inclusion bodies expressed in *Escherichia coli* and UCP 3 protein from yeast, as described by Cunningham *et al.* (27), were used as positive controls in immunoblot experiments. Following SDS-PAGE, resolved proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P^{SQ}; Millipore), as described by Cunningham *et al.* (27). Polyclonal antibodies to UCP 1 and UCP 3 peptides were raised in rabbits by Eurogentec (Herstal, Belgium) as detailed by Cunningham *et al.* (27). Commercial anti-UCP 1 (amino acids 145–159) and anti-UCP 2 (amino acids 144–157) rabbit antisera were purchased from Calbiochem. A goat antiserum to full-length rabbit UCP 1 protein was a gift from Dr. Daniel Ricquier, CNRS, Meudon, France. A rabbit antiserum specific for the β-subunit of F_1-ATP synthase from *Neurospora crassa* was a gift from Dr. Matt Harmey, Department of Botany, University College Dublin, Ireland. The antisera were all used at 1:1000 dilution. Following blocking and a 1-h primary antibody incubation, the blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000 dilution) in phosphate-buffered saline, 0.5% (v/v) Tween 20, 5% (w/v) milk powder for 1 h at room temperature. Blots were developed using an ECL detection system (Amersham Biosciences), and immunoreactions were visualized by exposure to Kodak X-Omat LS film.

GDP Binding Assay—Endogenous residual bound nucleotides were removed from isolated mitochondria using an anion exchanger (Dowex 21K) by the procedure described by Huang and Klingenberg (15). 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 h. Measurement of binding of tritiated GDP was performed by a modification of the procedure described by Scarpace *et al.* (28). Mitochondria (50 μg) were incubated with [^{3}H]GDP (0.1–6.0 μM, 11.0 Ci/mmol) and [^{14}C]sucrose (250 μCi/ml) for 15 min at 37 °C in the absence (total binding) and presence (nonspecific binding) of unlabeled GDP (1.5 mM). Specific binding was calculated from the difference between total and nonspecific binding. The Michaelis dissociation constant (K_D) and the maximal binding capacity (B_{max}), describing the saturable binding of [^{3}H]GDP, were obtained by fitting mean values for specific binding sites on the *y* axis and free radioligand concentrations used on the *x* axis by using the program "Sigma Plot," version 5 (SPSS Inc. Chicago). The data were fitted to a rectangular hyperbola by nonweighted, nonlinear least squares regression.

Oxygen Consumption by Nonphosphorylating Mitochondria—Oxygen consumption rates were measured using a Clark-type oxygen electrode as described by González-Barroso *et al.* (29). Mitochondria (1 mg/ml) were incubated at 37 °C in medium containing 120 mM KCl, 5 mM HEPES-KOH, pH 7.4, 1 mM EGTA, 16 μM fatty-acid free BSA, 5 μM atractyloside, 5 μM rotenone, and 1 μg/ml oligomycin. Nonphosphorylating (state 4) oxygen consumption rates were measured as the steady-state rates achieved on addition of 7.5 mM succinate (succinate-KOH, pH 7.4). The sensitivity of this state 4 oxygen consumption rate to GDP (1 mM) 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-type oxygen electrode was calibrated according to the procedure of Reynafarje *et al.* (30) assuming that 406 nmol of oxygen atoms were dissolved in 1 ml of incubation medium at 37 °C.

Purification of UCP 1 from Rat Thymus Mitochondria—Purification of UCP 1 was performed using a hydroxyapatite (HTP) column chromatography procedure as described by Lin and Klingenberg (31) with slight modification. Intact thymus mitochondria (8–10 mg) suspended in STE buffer (250 mM sucrose, 5 mM Trizma (Tris base), 2 mM EGTA, pH 7.4) were centrifuged at 22,600 × *g* for 10 min at 4 °C. The mitochondrial pellet was solubilized in 13% (v/v) octylpentaoctylethylene (octyl-POE) ether in STE (total volume ~500 μl) and incubated on ice for 10 min prior to loading the solubilized mitochondrial proteins onto an HTP column. The column was prepared by soaking 0.34 g of HTP in 10 ml of STE buffer, pH 7.4, at 4 °C, for 6 h prior to pouring it into a 1-ml column (Bio-Rad) (diameter 1 cm, length 6 cm). The soaking solution was removed from the column by centrifugation at 800 × *g* for 2 min at room temperature immediately prior to loading of the mitochondrial proteins. The HTP column, containing the solubilized mitochondrial proteins, was incubated at room temperature for 10 min (to denature the adenine nucleotide carrier) followed by 25 min of incubation at 4 °C. The column was then centrifuged at 800 × *g* for 2 min to remove the HTP eluate (UCP 1-enriched fraction), leaving behind the unwanted bound proteins. The protein concentration of the HTP eluate was determined (23), and the octyl-POE detergent was removed using a Bio-Bead (Bio-Rad) column. The Bio-Bead column was prepared by placing 2 ml of Bio-Beads (suspended in distilled/deionized H_2O) into a 2-ml syringe barrel and allowing them to settle. The H_2O was removed from the Bio-Beads by centrifugation at 800 × *g* for 2 min. The Bio-Beads were equilibrated in ~2 ml of STE buffer, pH 7.4, for 30 min prior to use and were further centrifuged at 800 × *g* for 2 min to remove excess STE buffer. The HTP eluate was loaded onto the Bio-Beads with gentle mixing and incubated for 2 h at 4 °C with periodic slight agitation using a vortex-type mixer. The Bio-Bead column was centrifuged at 800 × *g* for 2 min, and the protein eluate was collected. The protein concentration of this elute was determined, and ~5–10 μg of protein were analyzed by SDS-PAGE and colloidal Coomassie Brilliant Blue G-250 staining.

Sample Preparation for Mass Spectrometry—A 30–33-kDa colloidal Coomassie Brilliant Blue G-250-stained protein band was excised from a one-dimensional SDS-polyacrylamide gel (Fig. 6*A*) and was transferred to a 1.5-ml Eppendorf microcentrifuge tube (previously autoclaved and rinsed with 50% high pressure liquid chromatography grade methanol to remove any contaminants) containing 100 μl of 20% (w/v) ammonium sulfate. The excised band was de-stained for 2 days in several washes of 50% (v/v) methanol, 5% (v/v) acetic acid, dehydrated with acetonitrile, reduced for 30 min with 10 mM dithiothreitol at 56 °C, and alkylated for 30 min with 100 mM iodoacetamide at 45 °C as described previously (32). The carboxyamidomethylated protein band was digested overnight at 37 °C with 40 μl of 20 ng/μl sequencing grade,

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on September 17, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2005/03/23/M413315200.DC1.html

modified porcine trypsin according to the manufacturer's directions (Promega, Madison, WI). Peptides were extracted from the gel by using a series of elutions with 10% (v/v) formic acid. The resulting eluate pool was reduced to a final volume of 20 μl in a SpeedVac Concentrator (Savant, Hicksville, NY) and processed for mass spectrometry.

Peptide Mass Mapping—Peptide mass mapping was performed using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. A 5-μl amount of the trypsin-digested sample was desalted and concentrated using a micro ZipTip μ-C_{18} (C18 resin; P10, Millipore Corp., Bedford, MA). The peptide mixture was eluted from the ZipTip with 1 μl of the MALDI-TOF matrix, α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid, Sigma), and spotted onto a Voyager stainless steel MALDI plate (Applied Biosystems, Foster City, CA). An Applied Biosystems Voyager DE-STR mass spectrometer (Applied Biosystems, Foster City, CA) running in delayed extraction and reflectron mode was used to acquire MALDI-TOF data. Selected peptide masses were submitted to four on-line search algorithms that use peptide masses to identify proteins from primary sequence data bases as follows: MS-Fit (Protein Prospector software package; San Francisco, CA, prospector.ucsf.edu/), Mascot (Matrix Science, London, UK, www.matrixscience.com/), ProFound (Prowl, Rockefeller University, prowl.rockefeller.edu), and PeptideSearch (EMBL Bioanalytical Research Group, Heidelberg, Germany, www.mann.embl-heidelberg.de/GroupPages/PageLink/peptidesearch/Services/PeptideSearch/FR_PeptideSearchFormG4.html).

Liquid Chromatography/Tandem Mass Spectrometry—The remaining peptide digest was analyzed using LC-MS/MS. The peptide samples were first desalted and concentrated on a PepMap C18 precolumn and then separated on a PepMap C18 column (LC Packings-Dionex, The Netherlands) followed by tandem mass spectrometry using a PE Sciex QStar Pulsar i Quadrupole time-of-flight mass spectrometer (Applied Biosystems).

The independent data acquisition parameters were as follows: after a 1-s survey scan from 400 to 1500 *m/z*, peaks with signal intensity over 10 counts with charge state +2 to +4 were selected for MS/MS fragmentation followed by a 2-s MS/MS from 65 to 1800 *m/z* for the four most intense ions in the survey scan. Once an ion was selected for MS/MS fragmentation, it was put on an exclude list for 180 s to prevent that ion from being gated again. A 4-atomic mass unit peak window was used to avoid gating of masses from the same isotopic cluster during the survey scan. Keratin and porcine trypsin peak masses were put on an exclude list to prevent these ions from being selected for MS/MS analysis. Independent acquisition data were submitted to ProID (proprietary Applied Biosystems software) for bioinformatics analysis of public protein data base (NCBInr) and subsequent protein identification.

RESULTS

By using RT-PCR, UCP 1 transcripts were detected in rat thymus and BAT (Fig. 1, *lane 4* in each case). As shown previously (24), rat liver was negative for UCP 1 transcripts. The specificity of our primers for detection of transcripts encoding uncoupling proteins 1–3 in rat brown adipose tissue, known to express all of these transcripts, has been demonstrated previously (24). Although it was clear that transcripts for UCP 1 were present in rat thymus, it was important to know whether UCP 1 protein was expressed.

To that end we used an anti-UCP 1 polyclonal antibody (from Calbiochem). The specificity of the antibody (Calbiochem) is demonstrated in the supplemental figure. We showed that UCP 1 protein was present in mitochondria isolated from rat brown adipose tissue (Fig. 2*A, lane 1*) and mitochondria isolated from whole rat thymus of fed (*lane 2*) and fasted (*lane 3*) animals (and in thymocytes from fed and fasted rats; results not shown). UCP 1 was clearly expressed at higher levels in mitochondria from brown adipose tissue (Fig. 2*A, lane 1*). As expected, no UCP 1 protein was detected in equivalent amounts of mitochondria isolated from rat liver (Fig. 2*A, lane 4*). As a control for assessing protein loading, an antibody to the F_1 β-subunit of ATP synthase was used in immunoblots on the same mitochondrial preparations (Fig. 2*B*). Collation of data for three separate preparations and experiments demonstrated that there was no statistically significant difference in expression of the UCP 1 protein in mitochondria isolated from rat

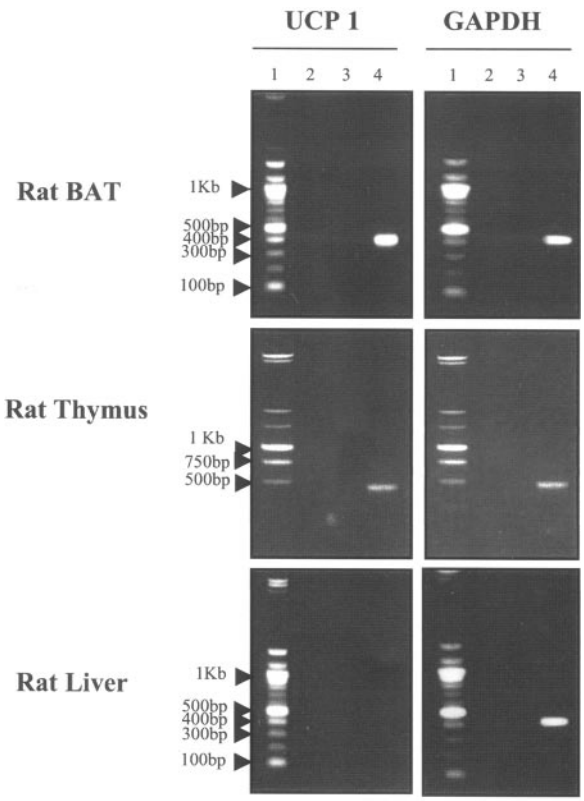


FIG. 1. **Detection of uncoupling protein 1 mRNA transcripts in various tissues.** *Lane 1*, DNA molecular markers (1 kb); *lane 2*, RT-PCR control reaction containing primers but no mRNA; *lane 3*, PCR control reaction containing primers but no cDNA; *lane 4*, RT-PCR product using primers to UCP 1 (399 bp) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (444 bp) for rat BAT, rat thymus, and rat liver.

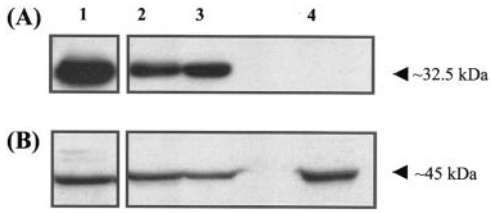
thymus when compared with the fed and fasted states (Fig. 2*C*; or rat thymocytes; results not shown). These results were confirmed using other anti-UCP 1 antibodies, namely a UCP 1-specific anti-peptide antibody characterized by Cunningham *et al.* (27) and also an antibody to the full-length UCP 1 (results not shown).

The anti-UCP 1 peptide antibody also detected UCP 1 in mitochondria isolated from the thymus of wild-type mice (Fig. 3, *lane 6*) but not in mitochondria from thymus of UCP 1 knock-out mice (Fig. 3, *lane 5*). The immunoblot also shows detection of UCP 1 in mitochondria isolated from BAT of wild-type mice (Fig. 3, *lane 8*) but not in mitochondria from BAT of UCP 1 knock-out mice (*lane 7*) as expected. No protein was detected using this antibody in mitochondria isolated from liver (Fig. 3, *lane 2*) or kidney (*lane 4*) of wild-type mice or liver (*lane 1*) or kidney (*lane 3*) of UCP 1 knock-out mice.

BAT can be present in the vicinity of the thymus. Therefore, a reasonable concern is that the detection of UCP 1 in mitochondria isolated from whole thymus could be due to BAT contamination of the thymus preparation. As BAT is visibly distinguishable from the translucent white thymus when present, it is easily removed (results not shown). However, to formally ensure there was no BAT contamination of the thymus, a single cell suspension of thymocytes was prepared from thymus from rats or mice. By using flow cytometry, it was confirmed that >99% of cells in the resulting thymocyte suspension from rats, and also mice, were positive for the thymocyte-specific marker, Thy-1 (Fig. 4*A*; data not shown). Consistent with the data from whole thymus (Figs. 1 and 3), both UCP-1 transcripts and UCP 1 protein were detected in this thymocyte preparation (Fig. 4, *B* and *C*).

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on September 17, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2005/03/23/M413315200.DC1.html



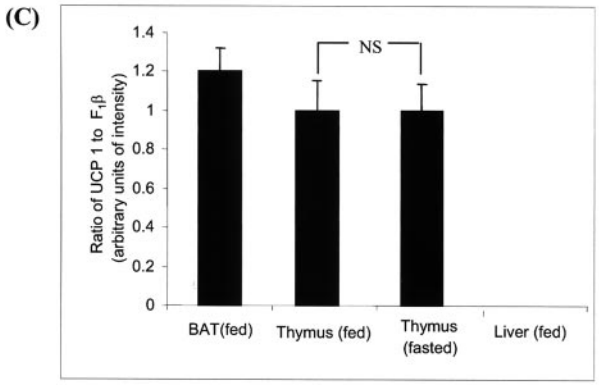


FIG. 2. **Detection of UCP 1 protein expression in mitochondria isolated from thymus of fed and fasted rats.** *A,* immunoblot using the anti-UCP 1 peptide antiserum (Calbiochem). *B,* immunoblot using the antibody to the β-subunit of the F_1-ATP synthase. *Lane 1*, BAT mitochondria isolated from fed rats. *Lane 2*, thymus mitochondria isolated from fed rats. *Lane 3*, thymus mitochondria isolated from fasted rats. *Lane 4*, liver mitochondria isolated from fed rats. *C,* the relative abundance of UCP 1 protein expression as a ratio to $F_1\beta$-subunit of the ATP synthase, as determined by densitometry, for three separate preparations. Data are expressed as mean ± S.E. No significant (*NS*) difference in UCP 1 expression was observed in thymus mitochondria in a comparison with the fed or fasted states ($p < 1.0$). In each lane 100 μg of protein was loaded.

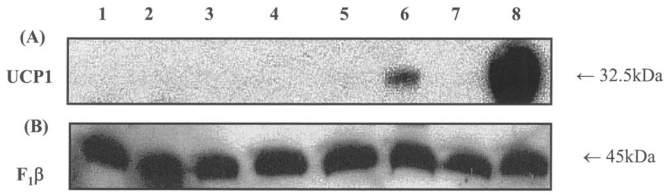


FIG. 3. **Detection of UCP 1 protein expression in mitochondria isolated from wild-type but not UCP 1 knock-out mice.** *A*, immunoblot using the anti-UCP 1 peptide antibody (Calbiochem) to mitochondria isolated from liver of UCP 1 knock-out (*lane 1*) and wild-type (*lane 2*) mice, kidney (*lane 3*) of UCP 1 knock-out and wild-type mice (*lane 4*) mice, mitochondria isolated from thymus of UCP 1 knock-out (*lane 5*) and wild-type mice (*lane 6*), mitochondria isolated from BAT of UCP 1 knock-out (*lane 7*) and wild-type mice (*lane 8*). *B*, an $F_1\beta$-subunit-specific antibody was used as a control for the presence of mitochondria. In *lanes 1–6*, 60 μg of protein was loaded, whereas in *lanes 7* and *8,* 20 μg was used.

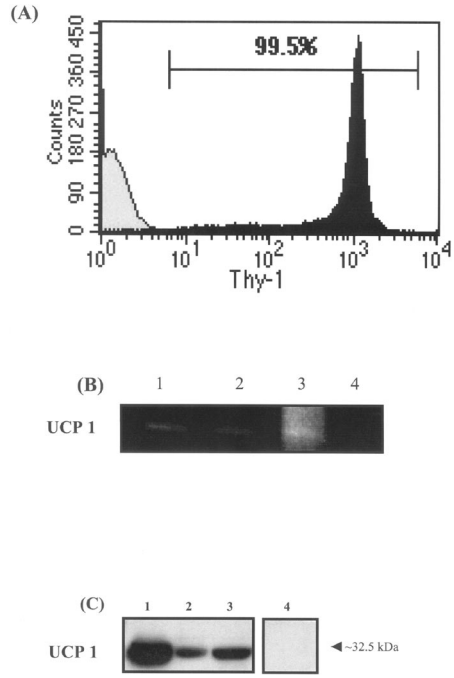


FIG. 4. **Detection of UCP 1 transcripts and protein in thymocytes.** *A,* flow cytometry analysis of the cell suspension prepared from the thymus of rats. Representative histogram image showing >99% of the thymocyte suspension are Thy-1-positive (*dark shading*) with gates set from isotype control IgG1 (*gray shading*). *B,* RT-PCR product using primers to UCP 1 (399 bp) in rat thymocytes (*lanes 1* and *2*). *Lane 3,* RT-PCR product using primers to UCP 1 (399 bp) in rat BAT; *lane 4,* PCR containing primers but no cDNA. *C,* immunoblot using the anti-UCP 1 peptide antiserum (Calbiochem) and immunoblot using the antibody to the β-subunit of the F_1-ATP synthase. *Lane 1*, brown adipose tissue (*BAT*) mitochondria isolated from fed rats; *lane 2,* thymocyte mitochondria isolated from fed rats; *lane 3,* thymocyte mitochondria isolated from fasted rats; *lane 4*, liver mitochondria isolated from fed rats. In each lane 100 μg of protein was loaded.

UCP 1 is known to contain a purine nucleotide-binding site that is accessible from the cytoplasmic side of the mitochondrial inner membrane. Thus, in the presence of atractyloside/carboxyatractyloside (which inhibits function and prevents purine nucleotide binding to the adenine nucleotide carrier), purine nucleotide binding to brown adipose tissue mitochondria can be used as a measure of UCP 1 abundance. However, experience from working with brown adipose tissue mitochondria taken from animals held at room temperature has shown that some of the purine nucleotide-binding sites in UCP 1 may be masked, *i.e.* purine nucleotides already bound. Unmasking can be achieved by treating mitochondria with Dowex ion-exchange resin that removes bound purine nucleotides (15). Unmasking manifests itself as an increase in the measured B_{max} of [^{3}H]GDP binding to brown adipose tissue mitochondria isolated from rats at room temperature (294 ± 29 pmol/mg (n = 3) to 394 ± 25 pmol/mg (n = 3), p = 0.048) as exemplified in Fig. 7*A*. The affinity of the binding site for GDP in brown adipose tissue mitochondria appeared not to be affected by Dowex treatment, as the K_D values are similar (0.6 ± 0.2 μM (n = 3) and 0.7 ± 0.2 μM (n = 3), respectively) (Fig. 5*A*). By using Dowex-treated mitochondria from various sources (to maximize the possibility of detecting binding), we showed that mitochondria containing UCP 1 from thymus could bind [^{3}H]GDP in a saturable manner with an equivalent K_D (0.7 ± 0.4 μM (n = 3)) and a B_{max} value (290 ± 46 pmol/mg (n = 3)) to mitochondria from BAT of rats that were kept at room temperature (394 ± 25 pmol/mg (n = 3) and 0.7 ± 0.2 μM (n = 3)) (Fig. 5*B*). Furthermore, in rat liver mitochondria, which contain no constitutively expressed UCPs (16, 27), rat skeletal muscle mitochondria (which contain UCP 3) (27), and rat spleen mitochondria, which contain UCP 2 (16), no saturable GDP binding was detected (Fig. 5*B*) (results not shown for spleen mitochondria). Thus the data show that the saturable GDP binding is because of the UCP 1 present in thymus mitochondria, as is the case for BAT mitochondria.

By having established that thymus mitochondria have a UCP 1-dependent GDP-binding site, it remained to be determined whether binding of the ligand could affect UCP 1 uncoupling function. Fig. 6*A* shows that the nonphosphorylating oxygen consumption rates (state 4; expressed as nanomoles of oxygen atoms/min/mg of mitochondrial protein) of non-Dowex-treated BAT mitochondria from rats kept at room temperature

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on September 17, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2005/03/23/M413315200.DC1.html

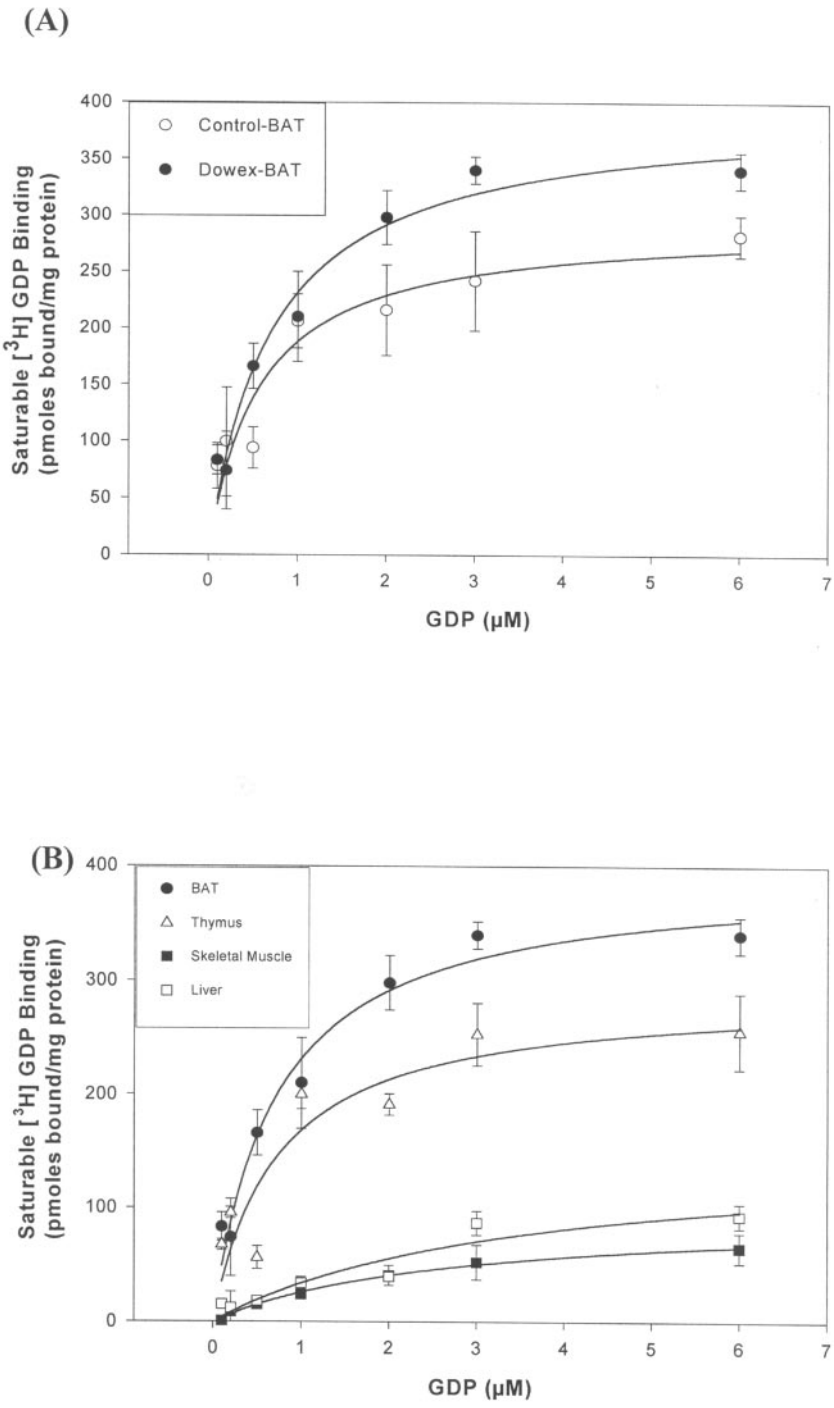


FIG. 5. **Measurement of GDP binding by thymus mitochondria.** Experiments were performed to determine B_{max} and K_D values for the GDP binding parameters. *A,* non-Dowex-treated (○) and Dowex-treated (●) BAT mitochondria from rat. *B,* Dowex-treated rat mitochondria from BAT (●), thymus (Δ), liver (□), and skeletal muscle (■). B_{max} values for non-Dowex-treated (294 ± 29 (n = 3)) and Dowex-treated (394 ± 25 (n = 3)) BAT mitochondria are significantly different (p = 0.048, unpaired Student's t test), whereas K_D values are similar (0.6 ± 0.2 μM (n = 3) and 0.7 ± 0.2 μM (n = 3) respectively). The B_{max} and K_D values for Dowex-treated thymus mitochondria are similar to those for Dowex-treated BAT mitochondria (290 ± 46 (n = 3) and 0.7 ± 0.4 (n = 3) respectively). The Michaelis dissociation constant (K_D) and the maximal binding capacity (B_{max}), describing the saturable binding of [^{3}H]GDP, were obtained by fitting mean values for specific binding sites on the y axis and the free radioligand concentrations used on the x axis by using the program Sigma Plot, version 5 (SPSS Inc. Chicago). The data were fitted to a rectangular hyperbola by nonweighted, nonlinear least squares regression. Data are mean ± S.E. from 3 independent experiments each performed in triplicate.

in the presence of inhibitors of the adenine nucleotide carrier and ATP synthase is inhibited by addition of 1 mM GDP (from 175 ± 6 to 128 ± 5; p = 0.0038). In turn, the oxygen consumption rate was significantly stimulated by nanomolar amounts of palmitate (~40 nM free fatty acid) to 190 ± 11 (p = 0.0088), close to the maximal uncoupled rate of 230.8 ± 14.2 induced by the addition of FCCP. In order to determine whether the UCP 1 in mitochondria (non-Dowex-treated) from thymus or thymocytes from rats kept at room temperature was functionally active, we measured the state 4 oxygen consumption rates under the same conditions, and we determined whether these rates were inhibited by GDP or activated by palmitate (buffered by defatted BSA). In addition, the maximal uncoupled oxygen consumption rate was determined. Fig. 6*B* shows that state 4 oxygen consumption rate by thymus/thymocyte mitochondria (64 ± 3) was significantly inhibited by GDP (34 ± 4) (p = 0.004) and was subsequently stimulated by the presence of a nanomolar concentration of palmitate (55 ± 6) (p = 0.04). The maximal uncoupled rate (+FCCP) is 107 ± 4 for thymus mitochondria. In contrast, state 4 oxygen consumption rates by (non-Dowex-treated) mitochondria from rat liver (39.4 ± 1.4; Fig. 6*C*), which contain no constitutively expressed UCPs, rat skeletal muscle mitochondria (101 ± 22), which only contain UCP 3 (Fig. 6*D*), and rat kidney mitochondria (65.5 ± 12.9) and rat spleen mitochondria (results not shown), both of which only contain UCP 2 (Fig. 6*E*), are GDP- and palmitate-insensitive. This insensitivity was not due to poor quality mitochondria preparations as the maximal oxygen consumption rates due to the uncoupler FCCP were 131 ± 12, 268.0 ± 16, 226 ± 19.0 for mitochondria from liver, skeletal muscle, and kidney, respec-

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on September 17, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2005/03/23/M413315200.DC1.html

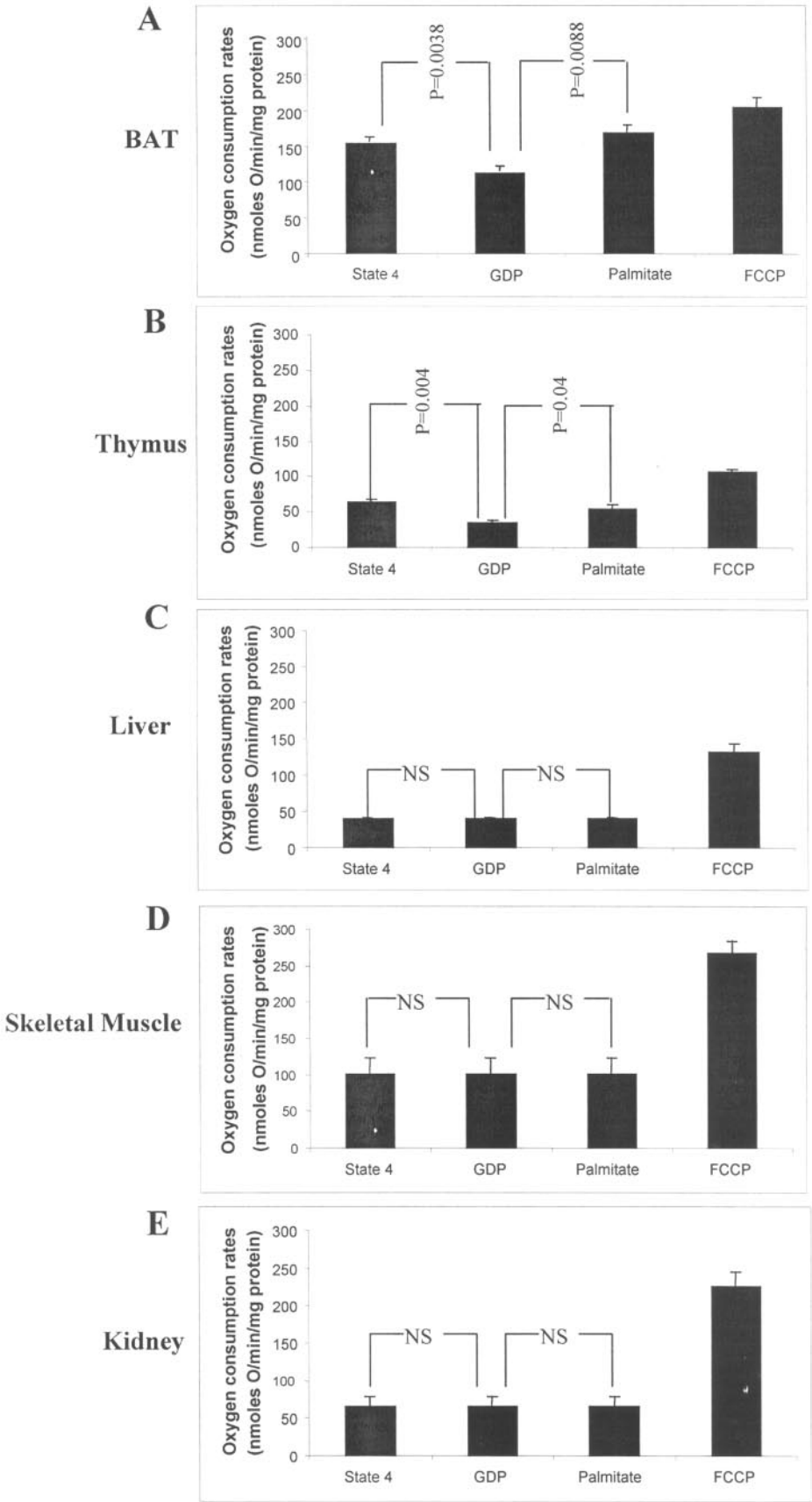


FIG. 6. **Measurement of state 4 oxygen consumption by thymus mitochondria in the presence of purine nucleotides and fatty acids.** Mitochondria (0.5 mg/ml; non-Dowex-treated) from BAT (*A*), thymus (*B*), liver (*C*), skeletal muscle (*D*), and kidney (*E*) of rats kept at room temperature were incubated in the presence of 120 mM KCl, 5 mM HEPES-KOH, pH 7.0, 1 mM EGTA, 7.5 mM succinate (K^+ salt), 16 μM de-fatted BSA, 5 μM rotenone, 1 μg/ml oligomycin, and 5 μM atractyloside. Steady-state oxygen consumption rates were then measured, following addition of 1 mM GDP, 64 μM palmitate (~40 nM free), and 40 nM FCCP. Data are shown as the mean ± S.E. from at least three independent experiments each performed in triplicate.

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on September 17, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2005/03/23/M413315200.DC1.html

tively. We also observed that the state 4 oxygen consumption rate was GDP- and palmitate-insensitive when thymus/thymocyte mitochondria were respiring on complex I substrates (glutamate plus malate or pyruvate plus malate, instead of succinate plus rotenone) (results not shown). This most probably reflects limitations in the supply of reducing equivalents to the mitochondria, as we also observed that addition of uncoupler failed to increase oxygen consumption in thymus/thymocyte mitochondria with these substrates (results not shown).

The immunoblot analyses and GDP binding assays led us to believe that there would be enough of the UCP 1 protein expressed in mitochondria from rat thymus to allow its purification. Hydroxyapatite chromatography procedures previously used for purification of UCP 1 from BAT (31) were adapted for rat thymus. After one-dimensional SDS-PAGE and staining with colloidal Coomassie Blue G-250, a distinct broad band of ~30–33 kDa (the expected mass of UCP 1) was observed (Fig. 7*A*). The band was excised, and tryptic peptides were prepared for analysis by mass spectrometry. The mass spectrum obtained using MALDI-TOF MS is shown in Fig. 7*B*. After internal calibration, base-line correction, and peak selection, 33 peptides were submitted for peptide mass fingerprinting. Searching the nonredundant data bases with the peptide masses (using all four search algorithms) identified mitochondrial brown fat UCP 1 from *R. norvegicus* as having the top Mowse score (best data base "hit"). Nine tryptic peptides and 15 nontryptic peptide matches (Table I) were identified, which provided 77% coverage of the UCP 1 protein (237 of 307 amino acids; Fig. 7*C*). This unequivocally identified rat UCP 1 as a major protein in the gel band. Most interestingly, when the error tolerance was relaxed to 0.5 Da, the data base search algorithms Profound and MS-FIT (but not the other two search algorithms) identified two presumably co-digested proteins (voltage-dependent anion-selective channel protein 1 and mitochondrial carnitine acylcarnitine translocase carrier protein). Peptides from these proteins were present in small amounts unlike those of UCP 1. These were also identified by LC-MS/MS (see next paragraph).

To refine the mass spectrometric identification of the isolated protein, tandem mass spectrometry (LC-MS/MS) was performed. Peptides were separated by liquid chromatography, gated, and fragmented, and the fragmentation data were used to search the data base. The results are shown in Table II. Four peptides (covering 15% of the protein) identified rat UCP. Other tryptic peptides were also sequenced, and data base searching identified three other mitochondrial proteins: voltage-dependent anion-selective channel protein 1 (VDAC 1), VDAC 2, and carnitine/acylcarnitine carrier protein. It is interesting that the masses of these proteins ranged from 32.4 to 33.5 kilodaltons, thus explaining why they were all present in the same protein band from the one-dimensional gel.

DISCUSSION

The aim of this study was to investigate the nature of the proton leak in thymus. As already mentioned, the specificity and selectivity of our primers for detection of transcripts encoding uncoupling proteins 1–3 have been demonstrated previously (24). In this study we clearly demonstrate that rat thymus contains UCP 1 transcripts. The results compliment our recent observations that rat thymus also contains transcripts for UCP 2 and UCP 3 (24). Our results, however, are in contrast to a previous study that failed to detect UCP 1 transcripts in rat thymus using Northern blot analysis (17). The inability to detect UCP 1 transcripts in that study may reflect a low abundance of UCP 1 mRNA in the thymus of rats kept at room temperature, which would only be detected by the more sensitive RT-PCR method used in this study. Previous studies on uncoupling proteins have emphasized the importance of determining whether mRNA levels reflect protein expression levels. For instance, by using Northern blot analysis, UCP 2 mRNAs were detected in many mammalian tissues (16), yet UCP 2 protein was only detected in a small subset of these tissues by immunoblot analysis (33). Discrepancies in UCP expression may also reflect difference in animal husbandry, *e.g.* basal room temperature, between different laboratories.

As with UCP 2, the detection of UCP 1 transcripts in thymus may not have necessarily reflected the presence of the UCP 1 protein. To that end, we characterized the UCP 1-peptide antibody (from Calbiochem) for it selectivity and sensitivity to UCP 1. Our data clearly demonstrate that the UCP 1-peptide antibody (Calbiochem) was selective for UCP 1 protein over UCP 2, UCP 3, and other mitochondrial transporters present in these mitochondria. Application of the UCP 1-peptide (Calbiochem) to mitochondria isolated from rat whole thymus detects UCP 1, a result that was reinforced by using a polyclonal anti-UCP 1 antibody produced for us by Eurogentec (27) and a polyclonal antibody to the full-length UCP 1 protein (a gift from Dr. Ricquier). Furthermore, we were able to detect UCP 1 in mitochondria isolated from mouse thymus but not in mitochondria isolate from UCP 1 knock-out mice. This observation further emphasizes the fact that we were detecting UCP 1 in the thymus and that the antibody was not cross-reacting with a mitochondrial protein present in thymus but not in other mitochondria.

We have ensured that the presence of UCP 1 in thymus is not because of BAT contamination of thymus. BAT can exist in the vicinity of the thymus, and when present it is easily discernible and excisable. Furthermore, in a highly processed cell suspension of thymocytes, which contained >99% Thy-1 (CD90)-positive lymphocytes, UCP 1 transcripts were detected by RT-PCR, and mitochondria isolated from these cells contain the UCP 1 protein. Future work will address why UCP 1 is present in thymocytes. In this respect, it is noteworthy that under conditions of acute starvation, which are known to diminish thymus function (34) and also reduce UCP 1 expression in BAT (35), we could detect no significant difference in the abundance of UCP 1 in mitochondria isolated from rat thymus. These data suggest that UCP 1 plays a different role in thymus than in BAT.

The presence of UCP 1 in rat thymus implied that mitochondria isolated from this organ might bind purine nucleotides. To improve the sensitivity of the purine nucleotide binding assay, we decided to treat the mitochondria to remove any endogenously bound purine nucleotide that might be present. Huang and Klingenberg (15) have shown that removal of any endogenously bound purine nucleotide can be achieved by treating the mitochondria with Dowex ion-exchange resin. We showed that Dowex-treated thymus mitochondria isolated from rats kept at room temperature could bind [^{3}H]GDP with similar affinity (K_D) and with a 75% B_{max} of that determined for BAT mitochondria. No saturable binding was detected in mitochondria from tissues such as spleen and kidney (which constitutively express UCP 2 protein), skeletal muscle (which constitutively expresses UCP 3), and liver (which does not express any UCPs) (16, 27, 36). We conclude that the [^{3}H]GDP binding observed in thymus mitochondria was due to UCP 1 protein.

The function of UCP 1 in BAT mitochondria is to generate heat by increasing metabolic flux in that tissue. BAT mitochondria have high oxygen consumption rates, *in vitro*, due to UCP 1 proton leak activity, an activity that is inhibited by purine nucleotides (*e.g.* GDP) and activated by long chain fatty acids (*e.g.* palmitate). Our evidence for the existence of UCP 1 protein in thymus mitochondria and the capability of those mitochon-

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on September 17, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2005/03/23/M413315200.DC1.html

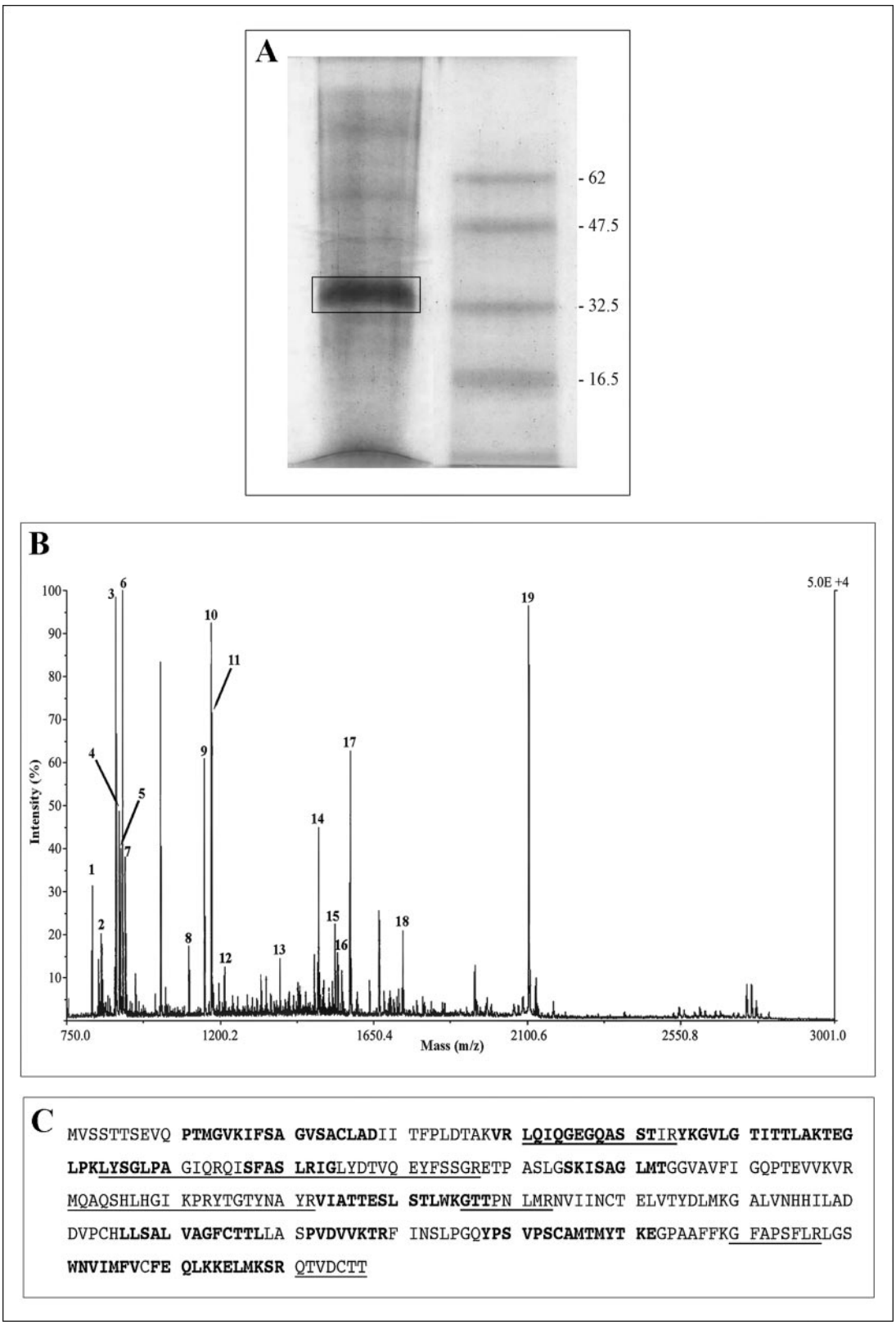


FIG. 7. **Identification of isolated rat thymus proteins by mass spectrometry.** *A*, one-dimensional gel electrophoresis analysis of purified UCP 1 from rat thymus mitochondria. The gel was stained with colloidal Coomassie Blue G-250. The broad band at 30–33 kDa was excised, and in-gel digestion with trypsin was performed prior to mass spectrometric analysis of tryptic cleavage products. Molecular mass markers are shown in the *right-hand lane*. *B*, the peptide mass spectrum obtained using MALDI-TOF MS. *C*, UCP 1 peptides identified by peptide mass fingerprinting. *Boldface*, tryptic peptide sequences. *Underlined*, nontryptic peptide sequences.

dria to bind GDP led us to determine whether thymus mitochondria had a UCP 1-catalyzed proton leak similar to that which had been observed for UCP 1 isolated from BAT. Although the state 4 oxygen consumption rates in thymus mitochondria were approximately a third that of BAT mitochondria, there was a UCP 1-catalyzed proton leak inhibited by GDP and

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on September 17, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2005/03/23/M413315200.DC1.html

TABLE I
MALDI-TOF identification of UCP 1 from peptide mass fingerprinting

Spectrum no.[a]	Peptide mass	Start amino acid	End amino acid	Peptide sequence	Fragment type
1[b]	824.474912	301	307	QTVDCTT-	Tryptic
2	850.448461	87	94	SFASLRIG	Nonspecific
3	894.493114	270	277	GFAPSFLR	Tryptic
4	905.497616	176	183	GTTPNLMR	Tryptic
	907.490526	115	123	SKISAGLMT	Nonspecific
5	908.492171	281	287	WNVIMFV	Nonspecific
6	913.461099	232	239	PVDVVKTR	Nonspecific
7	921.522377	85	92	QISFASLR	Tryptic
8	1108.521850	154	162	YTGTYNAYR	Tryptic
9	1153.651495	17	28	IFSAGVSACLAD	Nonspecific
10	1173.635104	69	79	EGLPKLYSGLP	Tryptic
11	1174.673948	74	84	LYSGLPAGIQR	Tryptic
12	1195.612911	217	228	LSALVAGFCTTL	Nonspecific
	1320.589168	154	164	YTGTYNAYRVI	Nonspecific
13	1374.692431	40	52	RLQIQGEGQASST	Nonspecific
14	1487.803679	41	54	LQIQGEGQASSTIR	Tryptic
	1502.814700	141	153	MQAQSHLHGIKPR	Tryptic
15	1536.851824	289	300	FEQLKKELMKSR	Nonspecific
16	1544.834032	66	80	AKTEGLPKLYSGLPA	Nonspecific
	1556.835564	90	102	SLRIGLYDTVQEY	Nonspecific
17	1580.820221	11	26	PTMGVKIFSAGVSACL	Nonspecific
	1707.832029	163	178	VIATTESLSTLWKGTT	Nonspecific
18	1734.866656	93	107	IGLYDTVQEYFSSGR	Tryptic
19	2103.272073	55	74	YKGVLGTITTLAKTEGLPKL	Nonspecific

[a] Masses without numerical designation were not abundant enough to be seen on the spectra without amplification of the base line.
[b] C terminus of UCP 1

TABLE II
LC-MS/MS identification of R. norvegicus mitochondrial proteins using peptide fragmentation data

Protein identification	GenBank™ accession no.	pI	Mass	No. peptides matched	Coverage of identified protein
			Da		%
Mitochondrial brown fat UCP 1	P04633	9.21	33458	4	15
VDAC-2	O9JI32	7.44	32353	3	11
VDAC-1	Q9Z2L0	8.35	32540	4	11
Mitochondrial carnitine/acylcarnitine carrier protein	CAA66410	9.55	33474	3	7

activated by nanomolar palmitate. As expected, there was no GDP-sensitive proton leak in liver mitochondria (which contain no UCPs), skeletal muscle mitochondria (which contain UCP 3), or spleen or kidney mitochondria (which contain UCP 2) (16, 36). We conclude that the GDP-inhibited and palmitate-activated proton leak measured in rat thymus mitochondria is not due to UCP 2 or UCP 3 but is due to the activity of UCP 1.

The detection of UCP 1 in rat thymus mitochondria using immunoblotting 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. We used procedures for purification of UCP 1 from BAT mitochondria (31), and we found that rat thymus mitochondria yielded a single broad 30–33-kDa band on a one-dimensional SDS-polyacrylamide gel, in accordance with the predicted molecular mass of UCP 1 (33,458 daltons). MALDI-TOF mass spectrometry results revealed that the predominant protein was uncoupling protein 1 from rat (GenBank™ accession number P04633). The abundant peptide masses showed that UCP 1 protein was by far the predominant molecule in the excised band from the one-dimensional gel. LC-MS/MS analysis confirmed the presence of UCP 1 peptides. LC-MS/MS analysis also revealed peptides from three other proteins, VDAC-1, VDAC-2, and mitochondrial carnitine/acylcarnitine carrier protein. These proteins have masses of 32,540, 32,353, and 33,474 Da, respectively; thus, it is not surprising that they migrate with UCP 1 (33,458 Da) as a single broad band on a one-dimensional gel. However, the isoelectric points of the four 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. Most interestingly, no native UCP 2 (or UCP 3) was detected in the purified 30–33-kDa band despite the fact that properly folded expressed UCP 2 (or UCP 3) passes through an hydroxyapatite column (38, 39). We can only assume that the abundance of UCP 1 protein in thymus is far greater than that for UCP 2 (or UCP 3).

The discovery of UCP 1 protein in rat thymus mitochondria has implications for understanding the bioenergetics, metabolism, and immune function of the thymus. Previous studies (20, 37) have described a proton leak (and a UCP 2-dependent proton leak) *in situ* in thymocytes, and therefore we might expect UCP 1 to play a major role in determining metabolic flux in the thymus. There is also evidence suggesting that UCP 1 plays a role in regulating superoxide production by mitochondria (5–8). The insensitivity of UCP 1 expression in thymus to starvation suggests a different role for UCP 1 in thymus than in BAT. The discovery of UCP 1 in rat thymus mitochondria has thus uncovered a new avenue for research into thymus bioenergetics, metabolism, development, and function.

Acknowledgments—We thank Dr. Daniel Ricquier (Centre de Recherche sur l'Endocrinologie Moleculaire et le Developpement, Meudon, France) for human UCP 2 and 3 clones; Prof. Jean-Paul Giacobino (Département de Biochimie Médicale, Université of Genève, Switzerland) for the mitochondria isolated from the skeletal muscle hind limb (gastrocnemius) of wild-type and UCP 3 knock-out mice; Dr. Jan Nedergaard (Wenner-Gren Institute, Stockholm University, Sweden) for the mitochondria isolated from brown adipose tissue of wild-type and UCP 1 knock-out mice; and Dr. Matt Harmey (Department of Botany, University College Dublin, Ireland) for the antibodies to the β-subunit of the F_1-ATP synthase.

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on September 17, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2005/03/23/M413315200.DC1.html

REFERENCES

1. Nicholls, D. G., and Locke, R. M. (1984) *Physiol. Rev.* **64,** 1–64
2. Nicholls, D. G. (2004) *Biochem. Soc. Trans.* **29,** 751–755
3. Nicholls, D. G. (1976) *Eur. J. Biochem.* **62,** 223–228
4. Ricquier, D., and Bouillaud, F. (1986) in *Brown Adipose Tissue* (Trayhurn, P., and Nicholls, D. G., eds) pp. 86–104, Edward Arnold Publishers, London
5. Echtay, K. S., Murphy, M. P., Smith, R. A. J., Talbot, D. A., and Brand, M. D. (2002) *J. Biol. Chem.* **277,** 47129–47135
6. Echtay, K. S., Roussel, D., St-Pierre, J., Jekabsons, M. B., Cadenas, S., Stuart, J. A., Harper, J. A., Roebuck, S. J., Morrison, A., Pickering, S., Clapham, J. C., and Brand, M. D. (2002) *Nature* **415,** 96–99
7. Echtay, K. S., Esteves, T. E., Pakay, J. L., Jekabsons, M. B., Lambert, A. J., Portero-Otin, M., Pamplona, R., Vidal-Puig, A. J., Wang, S., Roebuck, S. J., and Brand, M. D. (2003) *EMBO J.* **22,** 4103–4110
8. Murphy, M. P., Echtay, K. S., Blaikie, F. H., Asin-Cayuela, J., Cochemé, H. M., Green, K., Buckingham, J., Taylor, E. R., Hurrell, F., Hughes, G., Miwa, S., Cooper, C. E., Svistunenko, D. A., Smith, R. A. J., and Brand, M. D. (2003) *J. Biol. Chem.* **278,** 48534–48545
9. Skulachev, V. P. (1991) *FEBS Lett.* **294,** 158–162
10. Klingenberg, M., Echtay, K. S., Bienengraeber, M., Winkler, E., and Huang, S. G. (1999) *Int. J. Obes. Relat. Metab. Disord.* **6,** S24–S29
11. Garlid, K. D., Jabůrek, M., Ježek, P., and Vařecha, M. (2000) *Biochim. Biophys. Acta* **1459,** 383–389
12. Sundin, U., and Cannon, B. (1980) *Comp. Biochem. Physiol. B Comp. Biochem.* **65,** 463–471
13. Huang, S.-G., and Klingenberg, M. (1996) *Biochemistry* **35,** 16806–16814
14. Porter, R. K. (2001) *Biochim. Biophys. Acta* **1504,** 120–127
15. Huang, S. G., and Klingenberg, M. (1995) *Eur. J. Biochem.* **229,** 718–725
16. Ricquier, D., and Bouillaud, F. (2000) *Biochem. J.* **345,** 161–179
17. Nègre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Troly, M., Salvayre, R., Penicaud, L., and Casteilla, L. (1997) *FASEB J.* **10,** 809–815
18. Enërback, S., Jacobsson, A., Simpson, E. M., Harper, M.-E., and Kozak, L. P. (1997) *Nature* **387,** 90–94
19. Vidal-Puig, A. J., Grujic, D., Zhang, C. Y., Hagen, T., Boss, O., Ido, Y., Szczepanik, A., Wade, J., Mootha, V., Cortright, R., Muoio, D. M., and Lowell, B. B. (2000) *J. Biol. Chem.* **275,** 16258–16266
20. Buttgereit, F., Grant, A., Muller, M., and Brand, M. D. (1994) *Eur. J. Biochem.* **15,** 513–519
21. Chappell, J. B., and Hansford, R. G. (1972) in *Subcellular Components: Preparation and Fractionation* (Birnie, G. D., ed) pp. 77–91, Butterworth, London
22. Bhattacharya, S. K., Thakar, J. H., Johnson, P. L., and Shanklin, D. R. (1991) *Anal. Biochem.* **192,** 344–349
23. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem.* **87,** 206–210
24. Carroll, A. M., and Porter, R. K. (2004) *Biochim. Biophys. Acta* **1700,** 145–150
25. Tokunga, K., Nakamura, Y., Sakata, K., Fujimori, K., Ohkubo, M., Sawada, K., and Sakiyama, S. (1987) *Cancer Res.* **47,** 5616–5619
26. Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1998) *Electrophoresis* **9,** 255–262
27. Cunningham, O., McElligott, A. M., Carroll, A. M., Breen, E., Reguenga, C., Oliveira, M. E. M., Azevedo, J. E., and Porter, R. K. (2003) *Biochim. Biophys. Acta* **1604,** 170–179
28. Scarpace, P. J., Bender, B. S., and Borst, S. E. (1991) *Can. J. Physiol. Pharmacol.* **69,** 761–766
29. González-Barroso, M., Fleury, C., Bouillaud, F., Nicholls, D. G., and Rial, E. (1998) *J. Biol. Chem.* **273,** 15528–15532
30. Reynafarje, B., Costa, L. E., and Lehninger, A. L. (1985) *Anal. Biochem.* **145,** 406–418
31. Lin, C. S., and Klingenberg, M. (1980) *FEBS Lett.* **113,** 299–303
32. Kinter, M., and Sherman, N. E. (2000) *Protein Sequencing and Identification Using Tandem Mass Spectrometry,* pp. 147–165, John Wiley & Sons, Inc., New York
33. Pecqueur, C., Alves-Guerra, M.-C., Gelly, C., Levi-Meyrueis, C., Couplan, E., Collins, S., Ricquier, D., Bouillaud, F., and Miroux, B. (2001) *J. Biol. Chem.* **276,** 8705–8712
34. Kendall, M. D., and al-Shawaf, A. A. (1991) *Brain Behav. Immun.* **5,** 9–28
35. Boss, O., Muzzin, P., and Giacobino, J.-P. (1998) *Eur. J. Endocrinol.* **139,** 1–9
36. Echtay, K. S., and Brand, M. D. (2001) *Biochem. Soc. Trans.* **29,** 763–768
37. Krauss, S., Zhang, C. Y., and Lowell, B. B. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 118–122
38. Jekabsons, M. B., Echtay, K. S., and Brand, M. D. (2002) *Biochem. J.* **366,** 565–571
39. Jabůrek, M., and Garlid, K. D. (2003) *J. Biol. Chem.* **278,** 25825–25831

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on September 17, 2009