Accepted Manuscript

Crystal Structure of NAD-dependent *Peptoniphilus Asaccharolyticus* Glutamate Dehydrogenase Reveals Determinants of Cofactor Specificity

Tânia Oliveira, Santosh Panjikar, John B. Carrigan, Muaawia Hamza, Paul C. Engel, Amir R. Khan

PII: S1047-8477(11)00291-7
DOI: [10.1016/j.jsb.2011.10.006](https://doi.org/10.1016/j.jsb.2011.10.006)
Reference: YJSBI 6087

To appear in: *Journal of Structural Biology*

Received Date: 9 August 2011
Revised Date: 21 October 2011
Accepted Date: 24 October 2011

Please cite this article as: Oliveira, T., Panjikar, S., Carrigan, J.B., Hamza, M., Engel, P.C., Khan, A.R., Crystal Structure of NAD-dependent *Peptoniphilus Asaccharolyticus* Glutamate Dehydrogenase Reveals Determinants of Cofactor Specificity, *Journal of Structural Biology* (2011), doi: [10.1016/j.jsb.2011.10.006](https://doi.org/10.1016/j.jsb.2011.10.006)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Crystal Structure of NAD-dependent *Peptoniphilus Asaccharolyticus*
Glutamate Dehydrogenase Reveals Determinants of Cofactor
Specificity**

Tânia Oliveira^{¶*}, Santosh Panjikar[§], John B. Carrigan[¥], Muaawia Hamza[†],
Paul C. Engel[†] and Amir R. Khan[¶]

[¶] School of Biochemistry and Immunology, Trinity College Dublin, Dublin 2, Ireland

[†] School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

[§] Australian Synchrotron, 800 Blackburn Road, Clayton, VIC, Australia

[¥] CR UK Institute for Cancer Studies, University of Birmingham, Henry Wellcome Building for Biomolecular NMR Spectroscopy, Birmingham, United Kingdom.

*Current address: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (ITQB/UNL), Oeiras, Portugal

Author to whom correspondence should be addressed: ARK
Tel: +353 1 896 3868
email: Amir.Khan@tcd.ie

ABSTRACT

Glutamate dehydrogenases (EC 1.4.1.2–4) catalyse the oxidative deamination of *L*-glutamate to α -ketoglutarate using NAD(P) as a cofactor. The bacterial enzymes are hexamers and each polypeptide consists of an N-terminal substrate-binding (Domain I) followed by a C-terminal cofactor-binding segment (Domain II). The reaction takes place at the junction of the two domains, which move as rigid bodies and are presumed to narrow the cleft during catalysis. Distinct signature sequences in the nucleotide-binding domain have been linked to NAD vs. NADP specificity, but they are not unambiguous predictors of cofactor preferences. Here, we have determined the crystal structure of NAD-specific *Peptoniphilus asaccharolyticus* glutamate dehydrogenase in the apo state. The poor quality of native crystals was resolved by derivatization with selenomethionine, and the structure was solved by single-wavelength anomalous diffraction methods. The structure reveals an open catalytic cleft in the absence of substrate and cofactor. Modeling of NAD⁺ in domain II suggests that a hydrophobic pocket and polar residues contribute to nucleotide specificity. Mutagenesis and isothermal titration calorimetry studies of a critical glutamate at the P7 position of the core fingerprint confirms its role in NAD binding. Finally, the cofactor binding site is compared with bacterial and mammalian enzymes to understand how the amino acid sequences and three-dimensional structures may distinguish between NAD vs. NADP recognition.

Keywords: glutamate dehydrogenase, nicotinamide adenine dinucleotide, enzyme structure, X-Ray crystallography

Abbreviations: *Peptoniphilus asaccharolyticus* glutamate dehydrogenase (PaGDH), *Pyrobaculum islandicum* GDH (PyrGDH), bovine GDH (BovGDH), *Clostridium symbiosum* GDH (CsGDH)

Introduction

Glutamate dehydrogenases (GDHs) are found in nearly every organism and play an important role in nitrogen and carbon metabolism. In the oxidative deamination reaction, GDH links amino acid metabolism to the tricarboxylic acid (TCA) cycle by converting *L*-glutamate to 2-oxoglutarate (α -ketoglutarate), whereas the reductive amination reaction supplies nitrogen for several biosynthetic pathways (Smith et al., 1975). GDH belongs to the amino acid dehydrogenase enzyme superfamily, members of which members display differential substrate specificity and include valine, leucine and phenylalanine dehydrogenases (Britton et al., 1993). This enzyme superfamily has considerable potential in the production of novel non-proteinogenic amino acids for the pharmaceutical industry (Paradisi et al., 2007; Yamada et al., 1995). Phenylalanine dehydrogenase has also been widely exploited for diagnosis of phenylketonuria (Nakamura et al., 1996) and glutamate dehydrogenase similarly for measurement of 'blood urea nitrogen'.

Bacterial GDHs are homo-hexameric enzymes with 32 symmetry. Each polypeptide consists of an N-terminal Domain I that adopts an α/β fold with 5 (mixed) β -strands sandwiched between α -helices on both sides. The C-terminal NAD(P)⁺ binding domain II consists of a modified Rossmann fold with one of the β -strands in the reverse orientation (Baker et al., 1992b; Stillman et al., 1992). Domain I forms the majority of crystal contacts along the three-fold and two-fold axes, and the substrate-binding site is found in a deep groove at the junction of the two domains. Sequence identities vary from as little as 25% to over 90% among the bacterial enzymes, while the eukaryotic GDHs can be further divided into the hexameric and tetrameric classes. The hexameric mammalian enzymes are structurally similar to their bacterial counterparts, except that they contain an insertion of an α -helical nucleotide-binding

‘antenna’ near the C-terminus at the three-fold axis in the hexamer, which is important in the regulation of catalytic activity (Peterson and Smith, 1999). The tetrameric NAD⁺-dependent GDHs that are found in many fungi have a much larger subunit ($M_r = \sim 115,000$). The sequence clearly specifies a typical GDH subunit at one end but the remaining ~60% of the sequence is of unknown function (Britton et al., 1992).

Bacterial glutamate dehydrogenases vary according to their coenzyme preference: NAD⁺ specificity, NADP⁺ specificity, and those which have dual cofactor specificity. In dehydrogenases generally, the presence of a glutamate at a position adjacent to the 2'OH group of the coenzyme is reported to discriminate against NADP⁺ (Wierenga and Hol, 1983). This important residue (termed P7) was highlighted together with a glycine-rich motif, (GXGXXG) in a sequence fingerprint that determined coenzyme specificity in the widespread Rossmann fold of dehydrogenases. Enzymes that exclusively use NADP(H) usually have the characteristic glycine rich turn GSGXXA (termed P1 to P6) plus a smaller, uncharged residue at P7 with positively charged residues nearby, allowing for better interaction with the 2-adenosine phosphate. The glutamate dehydrogenase family has a further unique feature in that those enzymes binding NADP(H) only, such as the GDH of *Escherichia coli*, tend to possess an aspartate at P7, an amino acid which would not seem ideal in the adenosine phosphate binding pocket. *Peptoniphilus asaccharolyticus* GDH (PaGDH), for instance, has a glutamate (Glu243) at this position and has high specificity for NAD(H), with a k_{cat}/K_m approximately 1000-fold greater than for NADPH (Carrigan and Engel, 2007). Interestingly, replacement of Glu243 by the relatively conservative aspartate residue at P7 results in significant loss of enzymatic activity. Furthermore, mutagenesis to positively-charged residues (arginine, lysine) results in further loss of activity, although the enzyme no longer displays preference for NADH over NADPH (Carrigan and

Engel, 2007). To date, much of the structural understanding of GDHs has been based on the crystal structure/sequence analysis of *Clostridium symbiosum* GDH (CsGDH) in spite of the fact that this enzyme is an exception that violates the fingerprint rules (Baker et al., 1992a; Lilley et al., 1991). CsGDH differs in the residue at P6 (Ala) and P7 (Gly) despite the same or even stronger discrimination in favour of NADH over NADPH (Capone et al., 2011). In addition, the structures of several GDHs from hyperthermophilic archaeobacteria have been determined, including *Pyrobaculum islandicum* (PyrGDH) bound to NAD (Bhuiya et al., 2005), and the apo forms of *Thermococcus litoralis* (Britton et al., 1999) and *Thermococcus profundus* (Nakasako et al., 2001). Currently, there are no crystal structures of a bacterial enzyme in complex with NADP, but the structure of the dual-specificity bovine enzyme (BovGDH) has been determined in the presence of both NAD and NADP (Smith et al., 2001).

Here, we have determined the crystal structure of PaGDH in the apo state by X-ray crystallography. The poor diffraction quality of native crystals was overcome by generating selenomethionine-derivatized protein, and the structure was solved through the implementation of single anomalous diffraction (SAD) phasing methods in the software programme Auto-Rickshaw. The structure reveals an open conformation, and modeling of NAD⁺ into Domain II suggests that a hydrophobic pocket is critical for cofactor binding.

Materials and methods

Structure determination of PaGDH

Selenomethionine-derivatized PaGDH was expressed, purified and crystallised as described recently (Oliveira et al., 2010). Data were collected at beamline BM14 at the European Synchrotron Research Facility (ESRF) in Grenoble, France. Data collection statistics are shown in **Table 1**. The structure was solved using the SAD phasing protocol of Auto-Rickshaw (Blumenthal and Smith, 1975; Panjikar et al., 2009) and using the peak dataset from a selenomethionine protein crystal to 2.94 Å resolution. In the software pipeline, ΔF values were calculated using the program *SHELXC* (Sheldrick, 2008). Based on an initial analysis of the data, the maximum resolution for substructure determination and initial phase calculation was set to 3.4 Å. All 22 selenium positions present in the asymmetric unit were found using the program *SHELXD* (Sheldrick, 2008). The correct hand of the substructure was determined using the programs *ABS* (Hao 2000) and *SHELXE* (Sheldrick, 2008). The occupancy of all substructure atoms was refined and initial phases were calculated using the program MLPHARE (1994). The two-fold non-crystallographic symmetry (NCS) operator was found using the program RESOLVE (Terwilliger, 2000). Density modification, phasing extension and NCS-averaging were performed using program DM (Cowtan and Zhang, 1999) to 2.94 Å resolution. A partial model containing 656 residues out of the total 840 residues was built using BUCCANEER (Cowtan, 2006). The initial phases were improved by phase combination of experimental and model phases using the program SIGMAA (Read, 2006) in the MRSAD protocol of Auto-Rickshaw (Panjikar et al., 2009). The density modification was carried out using the program RESOLVE and the resultant phases were used to continue model building using the program BUCCANEER resulting in the placement of 838 residues in the electron density and

790 residues were docked into the sequence. The model was iteratively improved manually using COOT (Emsley and Cowtan, 2004) and subsequently by maximum-likelihood refinement in Refmac5 (Murshudov et al, 1999), with the inclusion of five TLS groups (Winn et al., 2001) for each monomer. The final model was refined at 2.94Å to $R_{\text{cryst}}/R_{\text{free}}$ of 24.7%/28.7%. Details of the phasing and refinement statistics are shown in **Table 2**.

Superpositions of structures and cofactor modelling

In order to enable structural comparisons, enzymes were superimposed using either Domain I or Domain II, separately. All modeling studies of cofactors involved superposition of Domain II, but no rigid-body docking or energy minimizations were performed so that the modeled complexes represent the enzyme conformations directly from the X-ray data. Although successful soaking of NAD^+ into crystals of CsGDH has been reported (Baker et al., 1992b), the co-ordinates are not available in the Protein Data Bank and could not therefore be used in this study. Glutamate dehydrogenase from the hyperthermophilic archaeon *Pyrobaculum islandicum* (PyrGDH) in complex with NAD^+ (PDB code 1v9l) was used to generate the approximate position of the cofactor in the bacterial enzymes PaGDH and CsGDH. Superposition of Domain II independently was essential for accurate positioning of the cofactor, since the relative rotation of the two domains varies significantly among bacterial enzymes. The root-mean-square deviation of 139 common C_α atoms (Domain II) of PaGDH and PyrGDH was 0.98Å using a secondary structure matching (SSM) algorithm. Between the more distantly related PaGDH and the mammalian enzyme BovGDH, the rmsd is 1.32Å for 149 common C_α atoms. Alignment of Domain II from CsGDH (residues 202-390) reveals an rmsd value of 1.72Å for 143 common C_α atoms. The deviations in structural

alignments are correlated to sequence similarities among the three structures, which range from 44% (PyrGDH/PaGDH) to 33% (CsGDH/PaGDH). Overall, the relatively low values are indicative of the structural conservation of the NAD/NADP binding pocket, thus enabling comparisons of their cofactor specificity.

Isothermal titration calorimetry (ITC)

Titration were carried out using a Nano ITC III from Calorimetric Science Corporation. The cell contained GDH at a typical concentration of 40 μ M to 80 μ M of binding sites (monomer). Enzyme concentration was determined at A_{280} using the extinction coefficient determined to be accurate according to previous protocols (Carrigan et al., 2005). In addition to the enzyme, and where appropriate, the cell also contained substrates to a concentration of 2mM. The syringe contained substrates/ligands at concentrations varying between 700 μ M to 5mM depending upon the experiment. For most experiments, a total of 20 injections (5 μ l each) were made over 350 seconds. In some cases, an initial pre-injection of 2 μ l volume was made and the result from this injection was not used for data analysis. The enzyme was prepared for ITC by being dialyzed extensively over two days, firstly against potassium phosphate buffer pH 7, which allowed for complete removal of ammonium sulphate while maintaining the enzyme in a very stable environment. The final step was to dialyse against Tris buffer at pH 7.6, which was also used as the experimental condition. The latter step was carried out in a small sealed chamber with 2M HCL also being stirred to stop the solution from picking up nitrogen from the atmosphere. The temperature of the cell was set at 296K. Blank titrations were done in the absence of the enzyme to determine heats of dilution and mixing. The control titrations were then subtracted from the experimental titrations prior to data analysis, and data were

analysed by the Bindwords™ software provided by Calorimetric Sciences Corporation. In most cases, the data could be successfully fitted to a binding model consisting of a single set of independent sites.

Results and discussion

The poor crystal quality of native PaGDH was resolved by crystallization of selenomethionine-derivatized protein. The crystals thus obtained were of higher diffraction quality, and the structure was subsequently solved using SAD methods. The quality of the model following the last cycle of refinement is shown in **Figure 1**. A section of the most well defined region (Domain I; **Figure 1a**) is pictured alongside the most poorly ordered region (Domain II; **Figure 1b**). However, the backbone and many of the side chains are clearly observed, even in the poorly defined sections of electron density maps.

The two molecules in the asymmetric unit of PaGDH are indistinguishable (rmsd for 395 aligned residues by SSM = 0.26Å), therefore discussions will be limited to molecule 'A' in the deposited co-ordinates. Dimerization is mediated by the first β -strand in Domain I, thus resulting in a continuous 10-stranded β -sheet spanning the two molecules. The biological hexamer is generated by application of the crystallographic 3-fold axis, perpendicular to the 2-fold non-crystallographic axis (**Figure 2**). Residues 274-294 were disordered and could not be modeled in the electron density maps. This segment of the protein is localized to the cofactor-binding segment and there are few interactions from Domain II with other subunits in the biological hexamer. The segment 274-294 constitutes α_{12} and β_J (CsGDH nomenclature), and will be discussed in more detail below.

Comparisons of the structure of PaGDH with CsGDH reveals that CsGDH contains an extra α -helix at the N-terminus (**Figures 3-4**). The nomenclature used for discussions of secondary structure corresponds to the original CsGDH structure in order to avoid confusion. Also, the CsGDH protein is the most intensely studied bacterial GDH, and therefore provides a convenient reference point for understanding structure and function. The overall structure of PaGDH resembles closely the NAD⁺-dependent CsGDH enzyme, which has been determined in both the apo state and glutamate-bound conformations (**Figure 3**). The substrate-binding cleft of PaGDH has an open conformation, and the relative orientation of the two domains resembles apo CsGDH. The angle relating three conserved residues in PaGDH - Pro78 in Domain I, Val353 in the helix α 15 (at the base of substrate-binding groove), and Phe221 in Domain II – was observed to be 72.8°. The equivalent residues in CsGDH – Pro96, Val377 and Phe238 – form an angle of 74.8° in the apo state, and 61.1° in the glutamate-bound state. In the NAD-bound structure of PyrGDH, these same conserved residues (Pro77, Val351, Met218) form an angle of 69.5°, thus revealing a relatively open catalytic cleft (**Figure 3**).

The last 3 α -helices of bacterial glutamate dehydrogenases (α 15- α 17 in CsGDH nomenclature) co-ordinate the inter-domain conformational changes during the reaction cycle (**Figures 3-4**). The pivot helix (α 16) is the second-to-last helix in the bacterial enzymes and packs against α 15, which binds to the substrate. Previous structural studies of CsGDH in open and closed conformations have revealed a critical role for the N-terminal segment of α 15, which lies at the base of the cleft and binds to glutamate (Stillman et al., 1999). The molecular mechanisms that couple substrate binding to global rotations of Domains I and II remain to be determined, but it is worthy noting that tandem glycine residues (Gly350-Gly351) in PaGDH reside at the N-terminal side

of $\alpha 15$ (not shown). This di-glycine motif is conserved in CsGDH, PyrGDH, and other bacterial and mammalian enzymes. Therefore, there is inherent flexibility adjacent to the pivot helix which may influence the observed conformational changes upon substrate binding, and closure of the cleft has been observed even in the absence of substrate and cofactor (Stillman et al., 1999). Domain closure in mammalian enzymes and associated catalysis is regulated by an α -helical ‘antenna’ (Peterson and Smith, 1999; Smith et al., 2001; Smith et al., 2002) that is inserted within the loop connecting $\alpha 15$ and the pivot helix ($\alpha 16$) of bacterial enzymes. These two additional anti-parallel α -helices are sites for allosteric regulation of catalytic activity by ADP and GTP.

Cofactor specificity in PaGDH and CsGDH

The structure of NAD⁺-dependent PaGDH may provide additional insight into the molecular basis for cofactor specificity. Structural determinants can be extrapolated from molecular modeling since domain II, at least partly, acts as a rigid body and appears to maintain some of the secondary structural elements required for cofactor binding. A chimeric enzyme consisting of Domain I from NADH-dependent CsGDH and Domain II from NADPH-dependent *E.coli* GDH (EcGDH) retained the cofactor specificity of the parent domain from EcGDH (Sharkey and Engel, 2009). Alignments of Domain II reveal a remarkable degree of complementarity for the positions of NAD (**Figure 5**). We modeled the cofactor at the active site by superimposing Domain II from the NADH-dependent enzyme of *P.islandicum* (PDB code 1v9l), which remains the only fully published bacterial GDH/cofactor complex.

In PaGDH, the Glu243 side chain (P7 residue) points toward the ribose so that the carboxylate group is within 3.2Å and 3.4Å of the 2'OH and 3'OH, respectively (**Figure 5**). The subsequent residue is Trp244, which stacks against one side of the

adenine ring of NAD^+ , while the other side of adenine is packed against Leu301. Thus, interactions with NAD^+ appear to be dominated by hydrophobic interactions and hydrogen bonds. A segment of PaGDH adjacent to the NAD^+ binding site, the region of $\alpha 11/\alpha 12$, is not observed in electron density maps and presumably disordered. This distal end of Domain II, at the extreme edge relative to the substrate-binding cleft, is the most heterogeneous part of GDH structures (**Figure 6**). In PyrGDH this region comprises the $\alpha 11$ -loop- $\alpha 12$ region (CsGDH nomenclature), and does not directly interact with NAD. However, it has been suggested that this region may help to distinguish cofactor preference (Bhuiya et al., 2005). The α -helical dipole at the N-terminus of $\alpha 10$, part of the highly conserved P-loop in nucleotide binding proteins, points towards the diphosphate moiety of NAD (**Figures 5,6**). This part of the cofactor marks the beginning of a highly conserved and pre-formed pocket that leads to the nicotinamide ribose moiety. Thus, a part of the NAD pocket is poised for binding, while the adenine ribose side of the pocket - responsible for specificity - reveals significant conformational diversity, and may close or become ordered upon binding its cognate cofactor (**Figure 6**).

Successful soaking of NAD^+ into the active site of CsGDH has been reported previously (Baker et al., 1992b). In that study, interpretable electron density was seen for 1/3 molecules in the asymmetric unit, and binding was accompanied by subtle side chain rotations. The adenine was bound *via* a pocket formed by Pro262 and Thr322, while the adenine ribose was accommodated in a pocket formed by Phe238 and Pro262. A hydrogen bond was observed between S260 and N3 of adenine. Since there is no deposited structure of CsGDH with the cofactor, we modeled the position of NAD^+ into CsGDH starting with PDB file 1bgv (glutamate complex, closed conformation). Our model reveals identical interactions at the adenine ribose side of NAD^+ relative to the

published experimental observations (**Figure 5**). The side chain of Ser260 is within 3.4Å of N3 and 3.2Å of N9 of adenine following alignments of Domain II, which suggests that the model has a fair degree of reliability. In addition, the side chain of Asn290 is within 3.6Å of the 2'hydroxyl of adenine ribose, which was also reported in the structure of the complex(Baker et al., 1992b). Overall, the loop connecting β H/ β I is close to the cofactor so that Pro262 stacks against the adenine ring. This stacking interaction is reminiscent of Trp244 in the NAD⁺-dependent PaGDH (**Figure 5**). Further reinforcing the similarities, the opposite face of adenine is packed against Thr322, which contributes both a hydrophobic surface and a hydrogen bond to the NH₂-group of adenine (3.2Å). As discussed previously, the interaction between Thr322 and the adenine was reported in the experimentally determined complex (Baker et al., 1992b). An identical theme is apparent in the structure of PyrGDH/NAD, in which the equivalent hydrophobic 'sandwich' for adenine is formed by Ile242 and Ile300 (**Figure 5**). In contrast to PaGDH and CsGDH, PyrGDH contains a proline insertion following strand β J (Pro284) that is within van der Waals distance of adenine (4.9Å). The residues involved in contacts with NAD in PyrGDH are marked with grey circles beneath the alignment (**Figure 4**).

Calorimetry and sequence motifs

In order to support the structural data, mutagenesis and isothermal titration calorimetry was performed to evaluate the strength of cofactor binding to PaGDH (**Figure 7, Table 3**). Cofactor was injected into wild-type (WT) and mutant proteins, and the heat of reaction was measured as a function of concentration. The binding of NADH to WT PaGDH involved favourable enthalpy with a K_d of 18.5 μ M. In the presence of substrate, the binding affinity was enhanced ($K_d = 0.87\mu$ M) due to a

favourable entropic term. This could imply a reduction of conformational entropy in the presence of glutamate, which would be consistent with the observation that generally the apo state of enzymes are open and more flexible than the closed state in the presence of reactants/products (Stillman et al., 1999).

In contrast to WT, the mutant E243K was severely crippled in its ability to bind to NADH. Even a modest mutation, E243D, hampers the catalytic ability of PaGDH (Carrigan and Engel, 2007), which can be explained by the disruption of hydrogen bonds with 2'OH and 3'OH groups of ribose. The inability of E243K to effectively switch to NADPH (**Table 3**) may be explained by the position of the P7 side chain, which would not be ideal for binding to the 2'-phosphate. Essentially, the Glu243-Trp244 would be held in place by stacking between the indole ring of Trp244 and the adenine of NAD⁺ (**Figure 5**). The importance of Trp244 is apparent from kinetic studies of W244S, which shows a 5-fold decrease in k_{cat}/K_m (Carrigan and Engel, 2007). Although there is no current structure of a bacterial NADP/GDH complex, BovGDH can utilize NADPH almost as well as NADH (Frieden, 1963). The conformation of BovGDH in complex with NADP is identical to the NAD-bound state (Figures 4,5), even within the heterogenous $\alpha 11$ -loop- $\alpha 12$ region (Smith et al., 2001). However, the 2'-phosphate makes additional contacts with Lys295 ($\alpha 11$), Glu275/Ser276 (P7-P8 residues), and Lys134 from Domain I (**Figure 5**). Therefore, favourable interactions with positively charged amino acids at key positions appear to be determinants of NADPH binding.

Concluding remarks

One of the key discriminants of nucleotide recognition is the P7 residue, which was identified in the sequence of P21-ras almost 30 years ago (Wierenga and Hol,

1983). In NAD-dependent PaGDH, a glutamate at the P7 position (Glu243) hydrogen bonds to 2'OH and 3'OH of ribose, due to its spatial disposition (**Figures 5,6**). The presence of a glycine at P7 can also be rationalized for the NAD-dependent CsGDH. Due to the lack of steric impediments from the P7-Gly residue, the side chain χ_1 of the P2 residue (Phe238) is rotated 180° relative to Phe220 of PaGDH and can form van der Waals contacts with the ribose sugar in CsGDH. Phenylalanine or a bulky residue is conserved in all NAD-dependent enzymes (**Figure 8**), and its conformation may be coupled to the P7 residue (glycine *vs.* acidic residue). Apparently conserved residues can mediate distinct roles in cofactor recognition depending on the structural context.

In summary, the molecular details underlying cofactor preference is a function of the conformation of conserved residues (e.g., Phe220 in PaGDH), and cannot be predicted from amino acid sequence alone. In contrast to hydrogen bonding *via* an acidic residue (Glu243 in PaGDH), the hydrophobic side chain of Phe238 stacks against the ribose near the 2'OH and 3'OH positions in CsGDH. These observations suggest caution in the interpretation of mutagenesis and kinetic studies without a structural framework for understanding the outcome. An emerging theme is the more hydrophobic nature of the adenine pocket in the NAD-dependent bacterial enzymes, relative to the more polar binding site in NADP-dependent or dual-specificity enzymes such as BovGDH. This pattern is repeated in the crystal structure of NADP-dependent *E.coli* GDH, which also has a relatively polar and positively charged binding pocket (Oliveira et al., *unpublished data*). Although our models of cofactor/GDH complexes are consistent with observed kinetic and mutagenesis studies, more detailed insight awaits the crystallization of bacterial enzymes with NAD⁺/NADP⁺ as well as intermediates in the catalytic pathway.

Accession numbers

Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 2yfq (PaGDH).

Acknowledgments

We would like to thank Dr. Hassan Belrhali of BM14 at the ESRF for his help in data collection. This work was supported by Science Foundation Ireland [grant number 03/IN.3/B371] to ARK.

References

- Baker, P.J., K.L. Britton, D.W. Rice, A. Rob, T.J. Stillman, 1992a. Structural consequences of sequence patterns in the fingerprint region of the nucleotide binding fold. Implications for nucleotide specificity. *J Mol Biol* 228, 662-71.
- Baker, P.J., K.L. Britton, P.C. Engel, G.W. Farrants, K.S. Lilley, D.W. Rice, T.J. Stillman, 1992b. Subunit assembly and active site location in the structure of glutamate dehydrogenase. *Proteins* 12, 75-86.
- Bhuiya, M.W., H. Sakuraba, T. Ohshima, T. Imagawa, N. Katunuma, H. Tsuge, 2005. The first crystal structure of hyperthermostable NAD-dependent glutamate dehydrogenase from *Pyrobaculum islandicum*. *Journal of molecular biology* 345, 325-37.
- Blumenthal, K.M., E.L. Smith, 1975. Alternative substrates for glutamate dehydrogenases. *Biochem Biophys Res Commun* 62, 78-84.
- Britton, K.L., P.J. Baker, D.W. Rice, T.J. Stillman, 1992. Structural relationship between the hexameric and tetrameric family of glutamate dehydrogenases. *European Journal of Biochemistry* 209, 851-859.
- Britton, K.L., P.J. Baker, P.C. Engel, D.W. Rice, T.J. Stillman, 1993. Evolution of substrate diversity in the superfamily of amino acid dehydrogenases. Prospects for rational chiral synthesis. *J Mol Biol* 234, 938-45.
- Britton, K.L., K.S. Yip, S.E. Sedelnikova, T.J. Stillman, M.W. Adams, K. Ma, D.L. Maeder, F.T. Robb, N. Tolliday, C. Vetriani, D.W. Rice, P.J. Baker, 1999. Structure determination of the glutamate dehydrogenase from the hyperthermophile *Thermococcus litoralis* and its comparison with that from *Pyrococcus furiosus*. *Journal of molecular biology* 293, 1121-32.
- Capone, M., D. Scanlon, J. Griffin, P.C. Engel, 2011. Re-engineering the discrimination between the oxidized coenzymes NAD(+) and NADP(+) in clostridial glutamate dehydrogenase and a thorough reappraisal of the coenzyme specificity of the wild-type enzyme. *FEBS J*.
- Carrigan, J.B., P.C. Engel, 2007. Probing the determinants of coenzyme specificity in *Peptostreptococcus asaccharolyticus* glutamate dehydrogenase by site-directed mutagenesis. *FEBS J* 274, 5167-74.
- Carrigan, J.B., S. Coughlan, P.C. Engel, 2005. Properties of the thermostable glutamate dehydrogenase of the mesophilic anaerobe *Peptostreptococcus asaccharolyticus* purified by a novel method after over-expression in an *Escherichia coli* host. *FEMS Microbiol Lett* 244, 53-9.
- CCP4, 1994. Collaborative Computational Project Number 4, 1994. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* 50, 760-3.
- Cowtan, K., 2006. The Buccaneer software for automated model building. 1. Tracing protein chains. *Acta Crystallogr D Biol Crystallogr* 62, 1002-11.
- Cowtan, K.D., K.Y. Zhang, 1999. Density modification for macromolecular phase improvement. *Prog Biophys Mol Biol* 72, 245-70.
- Emsley, P., K. Cowtan, 2004. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60, 2126-32.
- Frieden, C., 1963. Glutamate dehydrogenase. IV. Studies on enzyme inactivation and coenzyme binding. *The Journal of biological chemistry* 238, 146-54.
- Lilley, K.S., P.J. Baker, K.L. Britton, T.J. Stillman, P.E. Brown, A.J. Moir, P.C. Engel, D.W. Rice, J.E. Bell, E. Bell, 1991. The partial amino acid sequence of the NAD(+)-dependent glutamate dehydrogenase of *Clostridium symbiosum*: implications for the evolution and structural basis of coenzyme specificity. *Biochim Biophys Acta* 1080, 191-7.

- Nakamura, K., T. Fujii, Y. Kato, Y. Asano, A.J. Cooper, 1996. Quantitation of L-amino acids by substrate recycling between an aminotransferase and a dehydrogenase: application to the determination of L-phenylalanine in human blood. *Anal Biochem* 234, 19-22.
- Nakasako, M., T. Fujisawa, S. Adachi, T. Kudo, S. Higuchi, 2001. Large-scale domain movements and hydration structure changes in the active-site cleft of unligated glutamate dehydrogenase from *Thermococcus profundus* studied by cryogenic X-ray crystal structure analysis and small-angle X-ray scattering. *Biochemistry* 40, 3069-79.
- Oliveira, T.F., J.B. Carrigan, M.A. Hamza, M.A. Sharkey, P.C. Engel, A.R. Khan, 2010. Crystallization and preliminary structural analyses of glutamate dehydrogenase from *Peptoniphilus asaccharolyticus*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 66, 523-6.
- Panjikar, S., V. Parthasarathy, V.S. Lamzin, M.S. Weiss, P.A. Tucker, 2009. On the combination of molecular replacement and single-wavelength anomalous diffraction phasing for automated structure determination. *Acta Crystallogr D Biol Crystallogr* 65, 1089-97.
- Paradisi, F., S. Collins, A.R. Maguire, P.C. Engel, 2007. Phenylalanine dehydrogenase mutants: efficient biocatalysts for synthesis of non-natural phenylalanine derivatives. *J Biotechnol* 128, 408-11.
- Peterson, P.E., T.J. Smith, 1999. The structure of bovine glutamate dehydrogenase provides insights into the mechanism of allostery. *Structure* 7, 769-82.
- Read, R.J., 2006. Improved Fourier Coefficients for maps using phases from partial structures with errors. *Acta Crystallogr A* 42, 140-149.
- Sharkey, M.A., P.C. Engel, 2009. Modular coenzyme specificity: a domain-swapped chimera of glutamate dehydrogenase. *Proteins* 77, 268-78.
- Sheldrick, G.M., 2008. A short history of SHELX. *Acta Crystallogr A* 64, 112-22.
- Smith, J.E., J.D. Lane, P.A. Shea, W.J. McBride, M.H. Aprison, 1975. A method for concurrent measurement of picomole quantities of acetylcholine, choline, dopamine, norepinephrine, serotonin, 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, tryptophan, tyrosine, glycine, aspartate, glutamate, alanine, and gamma-aminobutyric acid in single tissue samples from different areas of rat central nervous system. *Anal Biochem* 64, 149-69.
- Smith, T.J., P.E. Peterson, T. Schmidt, J. Fang, C.A. Stanley, 2001. Structures of bovine glutamate dehydrogenase complexes elucidate the mechanism of purine regulation. *Journal of molecular biology* 307, 707-20.
- Smith, T.J., T. Schmidt, J. Fang, J. Wu, G. Siuzdak, C.A. Stanley, 2002. The structure of apo human glutamate dehydrogenase details subunit communication and allostery. *Journal of molecular biology* 318, 765-77.
- Stillman, T.J., P.J. Baker, K.L. Britton, D.W. Rice, H.F. Rodgers, 1992. Effect of additives on the crystallization of glutamate dehydrogenase from *Clostridium symbiosum*. Evidence for a ligand-induced conformational change. *J Mol Biol* 224, 1181-4.
- Stillman, T.J., A.M. Migueis, X.G. Wang, P.J. Baker, K.L. Britton, P.C. Engel, D.W. Rice, 1999. Insights into the mechanism of domain closure and substrate specificity of glutamate dehydrogenase from *Clostridium symbiosum*. *J Mol Biol* 285, 875-85.
- Terwilliger, T.C., 2000. Maximum-likelihood density modification. *Acta Crystallogr D Biol Crystallogr* 56, 965-72.
- Wierenga, R.K., W.G. Hol, 1983. Predicted nucleotide-binding properties of p21 protein and its cancer-associated variant. *Nature* 302, 842-4.
- Winn, M.D., M.N. Isupov, G.N. Murshudov, 2001. Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta crystallographica. Section D, Biological crystallography* 57, 122-33.
- Yamada, A., T. Dai, Y. Ohno, X.L. Huang, Y. Asano, 1995. Nucleotide sequencing of phenylalanine dehydrogenase gene from *Bacillus badius* IAM 11059. *Biosci Biotechnol Biochem* 59, 1994-5.

Figure Legends

Figure 1 Electron density maps of the refined model. Maps (2Fo-Fc) are shown at 1σ contour levels. (a) Section of the dimeric interface in the asymmetric unit. The two molecules are distinguished by the 'a' and 'b' suffixes. (b) NAD-binding region of Domain II in the vicinity of the P7 residue (Glu243).

Figure 2 Assembly of PaGDH into the biological hexamer with 32 symmetry. The asymmetric unit is the dimer with two-fold symmetry (yellow), mediated by β A of Domain I, that assembles into the hexamer *via* the 3-fold (horizontal) axis. Domain I mediates most of the inter-subunit contacts in the hexamer.

Figure 3 Domain flexibility of PaGDH, CsGDH and PyrGDH. Secondary structures are colour coded (red, α -helix; yellow, β -strand). The magnitude of inter-domain motion among the structures is highlighted by the angle formed by conserved residues in the domains and at the base of the substrate-binding groove. The disordered region of Domain II in PaGDH is marked. The CsGDH/glutamate panel is the enzyme/substrate complex, but the glutamate moiety is not shown. The NAD moiety in PyrGDH is a stick model with carbons in blue, to highlight the position of the cofactor. The lines relating the two domains are not shown for PyrGDH for cosmetic purposes, as one of them would lie on top of NAD. The PDB codes are 1hrd (apo CsGDH), 1bgv (CsGDH/glutamate complex) and 1v9l (PyrGDH/NAD complex).

Figure 4 Sequence alignments and annotation of NAD-dependent GDHs. The secondary structure cartoons correspond to PaGDH (top) and CsGDH (bottom), and

follow the convention of CsGDH names. Black dashed lines above PaGDH mark the region that is disordered in the crystal. The blue sphere (•) indicates the position of the P7 residue, while the grey spheres are residues that interact with NAD in the PyrGDH complex. Background green shading marks the boundaries of Domain I (1-186 in PaGDH), while the orange background highlights the last 3 α -helices (α 15- α 17) that mediate domain displacements during catalysis.

Figure 5 Cofactor specificity in glutamate dehydrogenases. Models were generated by superimposing Domain II of PyrGDH/NAD⁺ (residues 185-362) onto the equivalent regions of PaGDH and CsGDH (PDB code 1hrd). Panel PaGDH (yellow); adenine binding is dominated by hydrophobic residues in PaGDH (W244, L301). The P7 residue, Glu243, forms H-bonds to the 2' and 3'-hydroxyls of adenine ribose, while the Phe220 (P2 residue) points away from the sugar. Panel CsGDH (green); residues Pro262 and Thr322 form a pocket for the adenine component of NAD. In contrast to PaGDH, the P2-Phe residue (Phe238) packs against the ribose ring. Panel PyrGDH (grey); P7-aspartate (Asp241) H-bonds to 2'-hydroxy, and the Ile242/Ile300 form a hydrophobic pocket to accommodate the adenine base (PDB code 1v9l). Panel BovGDH (blue); the adenine ribose of NAD occupies a pocket with more polar character, relative to the bacterial enzymes (PDB code 1hwy). BovGDH is dual specificity, and can accommodate a 2'-phosphate group without significant conformational changes.

Figure 6 Conformational heterogeneity at the NAD binding site in GDHs. The region adjacent to the adenine ribose, which encompasses α 11/ α 12 and β H/ β I, is highly variable in sequence and conformation. The structures are PaGDH (yellow), CsGDH

(green, 1hrd), PyrGDH/NAD (grey), and BovGDH/NAD (blue, 1hwy). NAD from the PyrGDH complex is shown for reference as a stick model.

Figure 7 Raw and fitted data from isothermal titration calorimetry. The cofactor (5 μ L injections) was titrated into wild-type (WT) or mutant (E243K) PaGDH. The inset for each panel represents the raw heat per injection, and the graph of micro-Joules (μ j) vs. molar ratio is derived from the integrated heats. (a) 877 μ M of NADH was titrated into 40 μ M WT PaGDH in the presence of glutamate. (b) 118 μ M NADPH was titrated into 78 μ M E243K in the presence of oxoglutarate.

Figure 8 Multiple alignment of signature sequences of selected bacterial glutamate dehydrogenases. The NADP⁺-dependent enzymes are above, while the NAD⁺-dependent enzymes are grouped below. Residues of the ‘core fingerprint’ (P1-P7) of the co-factor binding site in glutamate dehydrogenases are denoted by numbers above the sequence alignment.

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 5

Figure 5

Figure 6

Figure 7

Figure 7

Figure 8

		123456	789
<i>E.coli</i>	234	RVS	SGSGNVAQYAIEKAMEFGARVITASDSSG-----TVVD--ESGFT
<i>S.enterica</i>	234	RVAV	SGSGNVAQYAIEKAMAFGARVVTASDSSG-----TVVD--ESGFT
<i>G.lamblia</i>	231	NVLL	SGSGNVAQFACEKLIQLGAKVLTFSDSNG-----TIVD--KDGFN
<i>C.glutamicum</i>	235	KIIV	SGSGNVATYAIEKAQELGATVIGFSDSSG-----WVHT--PNGVD
<i>S.cerevisiae1</i>	219	RVTIS	SGSGNVAQYAALKVIELGGTVVSLSDSKG-----CIIS--ETGIT
<i>C.sorokiniana</i>	248	RCLV	SGAGNVAQYCAELLLEKGAIVLSLSDSQG-----YVYE--PNGFT
<i>PsychrobacterTAD1</i>	230	KVLV	SGAGNVSLHAAEKATLIGAIVLTVSDSKG-----TIYD--AKGLN
<i>P.chrysogenum</i>	224	RVAIS	SGSGNVAQYAALKVIELGGSVISLSDSQG-----ALVLNGEEGSF
<i>N.crassa</i>	221	RVAL	SGSGNVAQYAALKLIELGATVVSLSDSKG-----ALVATGESGIT
<i>A.bisporus</i>	221	LVAIS	SGSGNVSOFTALKVIELGATVLSLSDSKG-----SLIS--EKGYT
<i>S.suis</i>	235	TVLIS	SGSGNVAQYAVQKATELGAKVISVSDSNG-----YIID--ETGID
<i>P.aeruginosa</i>	232	RVAIS	SGSGNVAQYAARKVMEMGGKVISLSDSEG-----TLYA--EAGLS
<i>C.symbiosum</i>	232	TVAL	AGFGNVAWGAACKLAELGAKAVTISGPDGY-----IYD--PEGIT
<i>P.asaccharolyticus</i>	214	KIAVQ	GFGNVGTFTVKNIERQGGKVCAIAEWDNRNEGNYALYN--ENGID
<i>B.fragilis</i>	230	TVAIS	GFGNVAWGAATKATELGAKVVTISGPDGY-----IYD--PNGIS
<i>P.gingivalis</i>	230	TLAIS	GFGNVAWGVAQKATELGIKVVTISGPDGY-----VYD--PDGIN
<i>E.caudatum</i>	224	TIAIS	GFGNVAFGAVLKAKQLGAKVVTISGPDGY-----IYD--ENGIN
<i>B.subtilis_rocG</i>	223	RIII	QGFNGSFLAKFMHDAGAKVIGISDANGG-----LYN--PDGLD
<i>B.subtilis_gudB</i>	226	RVVVQ	GFGNAGSYLAKFMHDAGAKVVGISDAYGG-----IYD--PEGLD
<i>C.difficile</i>	214	KIAVQ	GIGNVGSYTVLNCEKLGGTVVMAAEWCKSEGSYATYN--ENGLD
<i>V.vinifera</i>	208	TFVIQ	GFGNVGSWVARLIGERGKIIAVSDVTG-----AVKN--QNGLD
<i>P.islandicum</i>	212	TVAIQ	GMGNVGRWTAYWLEKMGAKVIAVSDINGVAYRKEGLN--VELIQ

Table I Crystallization and Data Collection

Crystallization	
protein	10mgmL-1,10mM HEPES, 100mM NaCl
reservoir	2M ammonium sulfate, 0.1M sodium cacodylate, 200mM NaCl, pH 6.5
Data collection	
Beamline	BM14, ESRF
Detector	Mar225 CCD
Space group	H32
Unit cell lengths (Å)	153.3, 153.3, 319.0
Unit cell angles (°)	90, 90, 120
Asymmetric unit	2 molecules
Wavelength (Å)	0.9786
Resolution (Å)	2.94 (3.01-2.94)
Total No. reflections	674,854
Unique reflections	58,934
Multiplicity	11.5(8.3)
Completeness (%)	99.8(97.8)
R _{merge} (%)	12.5(59.1)
I/σ all data	18.7(2.9)

Values in parentheses correspond to the statistics for the highest resolution.

Table 2 Phasing and Refinement Statistics**Phasing**

Resolution cut-off for initial phasing	3.4Å
Density modification/phase extend	2.94Å
SHELXD (CC _{all} /CC _{weak})	61.22/39.57
Se atoms sites in AU (obs/total)	20/22
Mean figure of merit	
MLPHARE (Overall/highest shell)	0.30 (0.22)
DM (Overall/highest shell)	0.62 (0.44)
After DM and two NCS averaging	
(Overall/highest shell)	0.69 (0.54)

Refinement statistics

No. unique reflections	29,106
Model (chain/residues)	
A	6-273, 295-421
B	5-273, 294-421
Ramachandran map (%)	
favour./allowed	98.8
outliers	1.2
R _{work} /R _{free} (%)	24.2/28.7
High res. shell	30.9/38.8

Non-hydrogen atoms

Protein	5,988
Sulphate	20
Water	6
Average isotropic <i>B</i> -factor (Å ²)	79.6
Average TLS <i>B</i> -factor	22.5

R.m.s. deviations

Bond lengths (Å)	0.012
Bond angles (°)	1.44
ML coordinate error (Å)	0.33

Values in parentheses correspond to the statistics for the highest resolution (3.01Å - 2.94Å).

Table 3 Isothermal titration calorimetry studies of cofactor binding to PaGDH

Ligand	Stoichiometry	K_d (μ M)	ΔH kJ/mol	ΔG (kJ/mol)	ΔS (J/mol·K)
NADH	0.9 (+/- 0.1)	18.5 (+/- 0.1)	-45.6	-32.6	-43.9
NADH (glutamate)	0.88 (+/- 0.1)	0.87 (+/- 0.03)	-23.0	-34.3	4.6
NADH (oxoglutarate)	0.53 (+/- 0.1)	14.7 (+/- 0.09)	-46.9	-33.1	-48.1
NADPH E243K oxoglutarate	N/D	>120	-5.0	-23.0	59.9

Thermodynamic parameters from the titrations of cofactor into PaGDH. Titrations were performed in the presence and absence of substrate/product, as indicated. Data were fit to a single-site model, as described in **Materials and methods**. Although the E243K mutant showed some affinity to NADPH, binding was weak and data were poorly fitted.