Crystal structure of a chimaeric bacterial glutamate dehydrogenase

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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 hexameric, arranged with 32 symmetry, and each polypeptide consists of an N-terminal substrate-binding segment (domain I) followed by a C-terminal cofactor-binding segment (domain II). The catalytic reaction takes place in the cleft formed at the junction of the two domains. Distinct signature sequences in the nucleotide-binding domain have been linked to the binding of NAD⁺ versus NADP⁺, but they are not unambiguous predictors of cofactor preference. In the absence of substrate, the two domains move apart as rigid bodies, as shown by the apo structure of glutamate dehydrogenase from *Clostridium symbiosum*. Here, the crystal structure of a chimaeric clostridial/*Escherichia coli* enzyme has been determined in the apo state. The enzyme is fully functional and reveals possible determinants of interdomain flexibility at a hinge region following the pivot helix. The enzyme retains the preference for NADP⁺ cofactor from the parent *E. coli* domain II, although there are subtle differences in catalytic activity.

1. 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 to several biosynthetic pathways (Smith *et al.*, 1975). GDH belongs to the amino-acid dehydrogenase enzyme superfamily (Britton *et al.*, 1993), which comprises valine, leucine and phenylalanine dehydrogenases. This enzyme superfamily has considerable potential in the production of novel nonproteinogenic amino acids for the pharmaceutical industry (Paradisi *et al.*, 2007; Yamada *et al.*, 1995), and phenylalanine dehydrogenase has been widely exploited for the diagnosis of phenylketonuria (Nakamura *et al.*, 1996).

Bacterial GDHs are homohexameric enzymes with 32 symmetry, and each subunit consists of an N-terminal domain I (which mediates hexamer assembly) and a C-terminal NAD(P)⁺-binding domain II with a Rossmann fold that has the direction of one strand reversed (Baker *et al.*, 1992; Stillman *et al.*, 1992). Domain I forms the majority of crystal contacts along the threefold and twofold axes, and the substrate-binding site is found in a deep groove at the junction of the two domains.

The eukaryotic GDHs can be further divided into hexameric and tetrameric classes. The hexameric mammalian enzymes, with a subunit molecular weight of approximately

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Data-collection statistics	
Space group	<i>P</i> 1
Unit-cell parameters (Å, °)	<i>a</i> = 100.2, <i>b</i> = 113.6, <i>c</i> = 109.3, $\alpha = 116.8$, $\beta = 101.5$, $\gamma = 104.0$
No. of molecules in asymmetric unit	6 [hexamer]
Wavelength (Å)	0.9792
Resolution (Å)	36.5–2.70 (2.79–2.70)
Completeness (%)	94.1 (93.5)
<i>R</i> _{merge} (%)	7.3 (42.2)
$\langle I/\sigma(I) \rangle$	10.8 (1.6)
Refinement statistics	
No. of unique reflections	108370
Residues in model	
Chain <i>A</i>	1–284, 289–448
Chain <i>B</i>	2–448
Chain <i>C</i>	2–448
Chain <i>D</i>	2–284, 289–448
Chain <i>E</i>	1–448
Chain <i>F</i>	2–448
Ramachandran plot statistics (%)	
Favoured/allowed	97.8
Outliers	2.2
<i>R</i> _{work} / <i>R</i> _{free} (%)	21.5/28.2 (28.4/36.9)
No. of non-H atoms	
Protein	20305
Water	305
Average <i>B</i> factor (Å ²)	72.5
R.m.s. deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.30
Maximum-likelihood coordinate error (Å)	0.41

55 kDa, 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 threefold axis in the hexamer, which is important in the regulation of catalytic activity (Peterson & Smith, 1999). Tetrameric GDHs have a subunit molecular weight of ~115 kDa, with the archetypal member being the NAD⁺-specific protein from *Neurospora crassa* (Veronese *et al.*, 1974).

Glutamate dehydrogenases vary according to their coenzyme preference: NAD⁺ specificity, NADP⁺ specificity or dual cofactor specificity (Engel, 2014). In dehydrogenases generally, the presence of an acidic residue in domain II, near

the 2'-position of the adenine ribose of the coenzyme [an OH in NAD(H), but substituted by a phosphate in NADP(H)], has been reported to discriminate against NADP⁺ (Scrutton *et al.*, 1990; Baker *et al.*, 1992). This important residue (termed P7) was highlighted together with a glycine-rich motif (GXG-XXG) in a sequence fingerprint that was said to determine 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–P6) plus a smaller, uncharged residue at P7 with positively charged residues nearby, allowing better interaction with the adenosine 2'-phosphate.

Bacterial GDHs can display significant relative rotations of domains I/II depending on the presence or absence of substrate/product at the active site. It is generally believed that the apo state of the enzyme has an open cleft which closes upon the binding of reactants. The epicentre of domain closure is believed to be the 'pivot helix' in bacterial and mammalian enzymes (Stillman *et al.*, 1999), which bridges the two domains and is positioned at the threefold axis in the hexamer. However, the structural determinants of domain flexibility have not been well characterized.

Here, we have solved the crystal structure of a chimaeric protein (CEC) that was engineered in three parts: domain I from the NAD⁺-dependent GDH of *Clostridium symbiosum* (CsGDH; residues 1–200), domain II from the NADP⁺-dependent GDH of *Escherichia coli* (EcGDH; residues 201–404) and the C-terminal helix again from CsGDH (residues 405–448) which re-enters domain I (Sharkey & Engel, 2009). We find that domain II maintains its structural and functional integrity independent of the hinge and domain I, as is borne out by the experimentally measured specificity of the chimaera (Sharkey & Engel, 2009).

2. Materials and methods

The chimaeric enzyme CEC was successfully expressed and purified as described previously (Sharkey & Engel, 2009). The protein was concentrated to 10 mg ml^{−1} and crystallized using the hanging-drop vapour-diffusion method at 18°C in 1.4 M ammonium sulfate, 0.1 M Tris–HCl pH 6.5–8.0, 10% dioxane.

Figure 1

Domain organization and cofactor preferences of bacterial glutamate dehydrogenases. The CEC protein is a chimaera built from CsGDH and EcGDH.

The crystals grew in one week to maximum dimensions of $0.2 \times 0.15 \times 0.15$ mm. Prior to X-ray data collection, the crystals were cryoprotected in a harvesting solution with 25% glycerol and were immediately flash-cooled in liquid nitrogen. X-ray diffraction data were collected on the NE-CAT 24-ID-C beamline at the Advanced Photon Source (APS), Argonne, Illinois, USA.

The diffraction data were integrated using the *HKL-2000* program suite and were scaled with *SCALA* from the *CCP4* program suite (Winn *et al.*, 2011). The CEC crystals belonged to space group *P1*, while the EcGDH crystals belonged to the orthorhombic space group *P2₁2₁2₁*, and both proteins crystallized with the biological hexamer in the asymmetric unit. The CEC structure was determined by molecular replacement using CsGDH (PDB entry 1hrd) and EcGDH (PDB entry 2yfg) as search models (Sharkey *et al.*, 2013; Yip *et al.*, 1995). After several failed trials of molecular replacement using different programs, a solution was obtained by running *Phaser* in two sequential steps (McCoy *et al.*, 2007). The substrate and cofactor domains were divided for the two models that were used (residues 1–200 of CsGDH and residues 201–447 of

EcGDH) and provided sequentially to *Phaser*. Initially, a substrate-domain search for six molecules was performed in *Phaser*. A single solution result was then used as an input for a second search on the cofactor domain. A single solution was obtained, with most of the model fitting the electron-density maps. Inspection of all chains through electron-density maps revealed an absence of or poorly defined density in some regions of the model. Poor definition resulted in chain breaks, even after iterative cycles of model building and refinement using *Coot* (Emsley *et al.*, 2010) and *REFMAC* (Murshudov *et al.*, 2011). *PROCHECK* from *CCP4* was used for structural analysis and model validation. Data-collection and refinement statistics are shown in Table 1.

3. Results and discussion

The overall hexameric assembly of CEC is common to all bacterial enzymes. The hexamers have 32 symmetry and CEC crystallized in the biologically relevant assembly with six molecules in the asymmetric unit. Both domains fold with a

Figure 2

Structures of bacterial glutamate dehydrogenases. The orientation is roughly aligned with a view down the pivot helix. CEC is a chimaeric construct composed of CsGDH (purple; domain I), EcGDH (green; domain II) and a C-terminal portion from CsGDH (part of the pivot helix and $\alpha 17$; pink). The C-terminus, which is found at the end of $\alpha 17$, is labelled 'C'. CsGDH, one of the parent enzymes of CEC, is shown in the closed conformation with a bound glutamate (orange spheres). The relative disposition of the two domains is emphasized by calculating the angle formed between conserved residues in the N-terminal domain (Asn134 in CEC), the base of the cleft (Asn430 in CEC) and the C-terminal domain (Ser287 in CEC).

central mixed β -sheet connected by α -helices, with domain II adopting a Rossmann nucleotide-binding fold.

In the numbering system for the chimaeric enzyme, domain I (residues 1–200 and 425–447) mediates the 32 symmetry contacts, while domain II (residues 201–424) is responsible for cofactor binding. The domain organization of CEC is shown in Fig. 1. Upon analysis of the aperture between domains I and II, the structure of CEC reveals that the six subunits (chains A–F) are in an open conformation. The relative degree of cleft opening can be quantified by measuring the angle formed by the base of the cleft (Asn430 in EcGDH) with two conserved residues in domains I and II of the enzyme (Lys136 and Arg289 in EcGDH; Fig. 2). In contrast to CEC, four of the six subunits in the solved structure of EcGDH are in a closed conformation (Sharkey *et al.*, 2013). The three molecules in the asymmetric unit of CsGDH have an open conformation, with the equivalent residues (Asn133, Arg289 and Asn431) having apertures of 32, 28 and 34°, respectively. Typical values for the closed conformation range from 19.5° (PDB entry 1bgv; CsGDH–glutamate complex; Stillman *et al.*, 1993) to between 17.3 and 23.9° for the closed ‘apo’ forms of EcGDH.

Modelling of NADP⁺ in domain II of CEC was performed following alignment of the equivalent domain from the bovine GDH–NADP⁺ complex (Fig. 3; Peterson & Smith, 1999). The model reveals a preformed cofactor-binding site, with potential hydrogen bonds between Ser263 and the 2'-phosphate (3.6 Å), as well as between Ser239 and the 3'-OH (2.5 Å). Asp262 is pointing away from the negatively charged phosphate and forms a hydrogen bond to the backbone NH of Val292, thus stabilizing the first turn of an α -helix (Sharkey *et al.*, 2013).

In summary, the model is consistent with the recently published structure of EcGDH and confirms that cofactor specificity is encoded by domain II of the bacterial enzymes (Sharkey & Engel, 2009). Nevertheless, there are differences

Figure 3

Modelling of the substrate-binding cleft of CEC. The structure of bovine GDH in complex with NADP⁺ (PDB entry 1hwz; Smith *et al.*, 2001) was used to model the cofactor into domain II of CEC. The root-mean-square (r.m.s.) deviation for 164 equivalent C α atoms (residues 206–389 of CEC) was approximately 1.95 Å, as determined by secondary-structure matching (Krissinel & Henrick, 2004). Several hydrogen bonds are predicted from modelling (dashed lines) and the two rings (adenine and nicotinamide) are able to pack nicely against hydrophobic residues in the homology-modelled complex.

in the kinetic properties of CEC relative to its parent enzymes EcGDH and CsGDH. Among the surprising observations is that CEC is able to catalyse several-fold higher reaction rates in both directions than either of the parent enzymes (Sharkey & Engel, 2009). These differences are likely to be associated with the conformational transitions involving domains I and II during the catalytic cycle. In the CEC structure, the pivot loop of CsGDH has been transplanted onto the C-terminus of domain II of EcGDH. It is possible that the open conformation of CEC observed in all six subunits of the hexamer may be derived from the parent CsGDH enzyme.

Another difference relates to cooperativity in the response to the substrate glutamate. This cooperativity is very marked in the clostridial enzyme (Wang & Engel, 1995), but only at high pH. For CEC, cooperativity is evident at all pH values tested (Sharkey & Engel, 2009), but the structure has thus far yielded no clues to explain this difference. In this context, a glaring omission remains the absence of a structure of the high-pH ‘inactive’ conformation of CsGDH. The kinetics of domain closure, efficiency in catalysis, cooperativity and pH dependence of the reaction are likely to be complex functions of interdomain interactions and are as yet difficult to attribute to individual residues.

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