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Crystallization and preliminary structural analyses of glutamate dehydrogenase from *Peptoniphilus asaccharolyticus*

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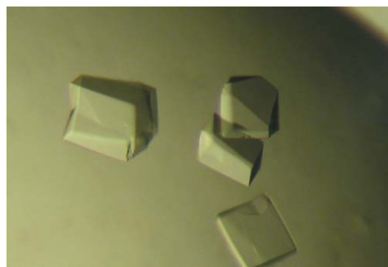
Glutamate dehydrogenase (EC 1.4.1.2–4) from *Peptoniphilus asaccharolyticus* has been expressed as a selenomethionine-derivatized recombinant protein and diffraction-quality crystals have been grown that are suitable for structure determination. Preliminary structural analyses indicate that the protein assembles as a homohexameric enzyme complex in solution, similar to other bacterial and mammalian enzymes to which its sequence identity varies between 25 and 40%. The structure will provide insight into its preference for the cofactor NADH (over NADPH) by comparisons with the known structures of mammalian and bacterial enzymes.

1. Introduction

Glutamate dehydrogenase (GDH) is a widely distributed enzyme in living organisms and plays an important role in nitrogen and carbon metabolism (Smith *et al.*, 1974; Baker *et al.*, 1992). This enzyme provides a key link between catabolic and biosynthetic pathways. It belongs to the amino-acid dehydrogenase superfamily, the members of which are responsible for the oxidative deamination of L-amino acids to their corresponding α-keto acids (Britton *et al.*, 1993). The members of this superfamily are able to metabolize glutamate, valine, leucine and phenylalanine. There is considerable interest in exploiting these enzymes to synthesize novel nonproteinogenic amino acids and also for use as diagnostic reagents to monitor the serum levels of amino acids which accumulate in a range of metabolic diseases. In addition to the widely distributed family of hexameric GDHs with a subunit of ∼50 kDa, there is also a family of NAD^+-dependent tetrameric GDHs with a much larger M_r of about 115 000. There is little sequence homology between these two classes of enzyme and no crystal structure is currently available of a tetrameric enzyme. Therefore, the biological role of the tetrameric GDHs and the molecular determinants that govern the nature of the oligomeric assembly remain unknown (Britton *et al.*, 1992).

Glutamate dehydrogenase is responsible for the first step in glutamate fermentation in some anaerobic bacteria (Buckel & Barker, 1974). It catalyses the oxidative deamination of glutamate to 2-oxoglutarate using NAD^+/$NADP^+$ as a cofactor. The glutamate dehydrogenase from *Peptoniphilus* (formerly *Peptostreptococcus*) *asaccharolyticus* (PaGDH) has been characterized extensively, but its structure has not been determined (Snedecor *et al.*, 1991; Carrigan & Engel, 2007). The presence of an acidic residue at the P7 position, adjacent to the 2′-OH group, typically discriminates against $NADP^+$ (Wierenga & Hol, 1983). PaGDH contains a P7 glutamate and has high specificity for NAD(H), with a k_{cat}/K_m for NAD^+ that is approximately 1000-fold greater than that for $NADP^+$ (Carrigan & Engel, 2007). In contrast, clostridial GDH, the structure of which has been solved (Stillman *et al.*, 1999), should have glutamate at this position but instead has Gly and is very strongly NAD^+-dependent. Furthermore, *Escherichia coli* GDH, the structure of which has not been refined (Korber *et al.*, 1993), is $NADP^+$-dependent but has Asp at the P7 position. A further puzzle concerns the P6 residue, which occupies the last position in the so-called 'core fingerprint' G-G-G/A (Wierenga *et al.*, 1985); in NAD^+-dependent enzymes this last residue is expected to be glycine, allowing a tight turn between a β-strand and



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an α-helix in the Rossmann fold. In NADP$^+$-dependent enzymes this position is usually occupied by Ala. PaGDH obeys this rule, with Gly at P6, but the clostridial enzyme with the same specificity contains Ala. These observations suggest that discrimination between NAD$^+$ and NADP$^+$ is more complex than anticipated. Further structural studies, in particular a comparison of the enzyme–coenzyme binary complexes, would enable an understanding of how these enzymes achieve selectivity and permit comparisons with the properties of related enzymes and the superfamily of amino-acid dehydrogenases.

Here, we report the crystallization and preliminary structural studies of glutamate dehydrogenase from *P. asaccharolyticus*.

2. Materials and methods

2.1. Cloning, expression and purification

P. asaccharolyticus was purchased from DSMZ GmbH, Braunschweig, Germany and cultured under anaerobic conditions in cooked-meat medium. The *PaGDH* gene was amplified from a genomic DNA preparation from this culture using the oligonucleotide primers 5′-AAA**GGATCC**ATGACAGATACACTTAATCCGTTAGTAGCGG-3′ and 5′-AAAAA**GTCGAC**GGACTACCTAAGTAGTCCCTTAATTTAGC-3′ and was subsequently cloned into the *Bam*HI and *Sal*I sites of the expression vector ptac-85 using standard techniques without any affinity tags to give residues 1–421 of PaGDH.

Native and SeMet-derivatized proteins were expressed and purified in *E. coli*. For the native protein, the vector was transformed into BL21 (DE3) cells and grown in 2×YT medium. For the SeMet protein, prB834 (DE3) cells were transformed and grown in minimal medium (Molecular Dimensions, UK) supplemented with 100 mg l^{-1} L-SeMet. Native and SeMet protein expression was induced upon reaching an OD_{600} of 0.7 by incubating the cells with 0.5 m*M* IPTG (final concentration) for 3–4 h at 310 K with vigorous shaking. Harvested cells were suspended in 0.1 *M* potassium phosphate buffer pH 7, sonicated and centrifuged to remove cell debris. The overexpression resulted in a cell-free extract in which 8–10% of the total protein was PaGDH. The first purification step, which exploited the remarkable thermostability of this mesophilic enzyme, was a 10 min incubation at 343 K, which resulted in around fourfold purification without loss of activity. The supernatant after centrifugation was loaded onto a Q-Sepharose anion-exchange column (GE Healthcare) in a cold room. After extensive washing with 0.1 *M* potassium phosphate pH 7, the column was eluted with a gradient of 0–1 *M* NaCl in the same buffer. This simple two-step procedure gave a main fraction of homogeneous GDH with a 95% yield. However, in order to produce protein of sufficient purity for crystallization a third step was introduced, namely hydrophobic interaction chromatography on butyl-Sepharose (GE Healthcare). The protein was loaded in 0.1 *M* potassium phosphate buffer containing 2.5 *M* ammonium sulfate and the column was extensively washed with this solution before eluting with a decreasing gradient of 2.5–0 *M* ammonium sulfate in the same buffer, which gave a main fraction of pure GDH.

In order to confirm the oligomeric state of the protein, the molecular weight was estimated using static light scattering (miniDAWN, Wyatt Corp) coupled with gel filtration (Superdex-200 10/300 GL, GE Healthcare), confirming that PaGDH is a hexamer with a molecular mass of 3.19×10^5 Da.

2.2. Crystallization

Native and SeMet-derivatized proteins were concentrated to 10 mg ml^{-1} using an iCON concentrator with a 9000 molecular-weight cutoff (Thermo Fisher Scientific, Ireland) in 10 m*M* Tris–HCl pH 7.0 buffer. Initial screening of crystallization conditions was performed using a Mosquito robot (TTP Labtech, UK) and 96-well commercial screens. After initial hits were obtained, crystals were optimized by the hanging-drop vapour-diffusion method at 293 K using 500 µl reservoir solution in 24-well Linbro plates. Early crystallization studies focused on native protein, but the crystal size and morphology were extremely poor. Therefore, our attention switched to SeMet-derivatized protein in an attempt to obtain higher quality crystals for structural studies. Varying ratios of SeMet protein and precipitant solutions were used and crystals with differing morphologies and sizes appeared within 1–3 months. The crystal morphology was dependent on precipitants such as ammonium sulfate and polyethylene glycol (PEG 6K) together with buffers such as MES, sodium cacodylate and HEPES (pH range 6–7.5). Despite the wide variety of conditions, the best crystals, with a cubic shape and varying dimensions, appeared upon mixing 2 µl protein solution with 1 µl 2.0 *M*

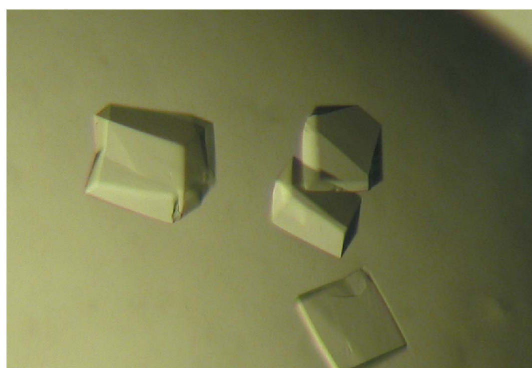

Figure 1
SeMet PaGDH crystals grown by the hanging-drop vapour-diffusion method to dimensions of 0.1 × 0.1 × 0.1 mm.

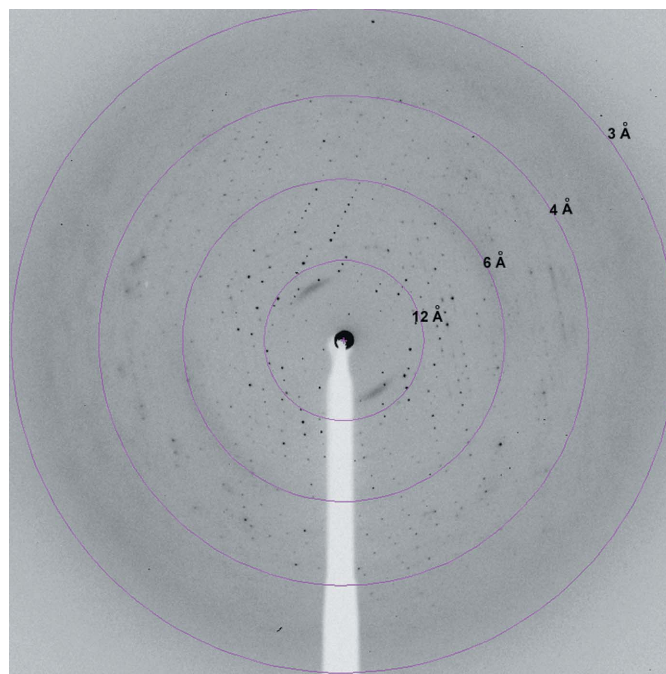

Figure 2
X-ray diffraction image of the SeMet PaGDH crystal from which the current best data set was collected. The image was obtained on beamline BM14 at the ESRF.

ammonium sulfate, 0.1 *M* sodium cacodylate pH 6.5, 200 m*M* NaCl (Fig. 1). To avoid ice formation, crystals were soaked in crystallization solution containing 25% glycerol and immediately cryocooled in liquid nitrogen.

2.3. Data collection and processing

Most crystals diffracted poorly, but one crystal was found for which an X-ray data set could be collected to 3.5 Å resolution on beamline BM14 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Analysis of the diffraction pattern revealed diffuse scattering (Fig. 2), which could indicate lattice disorder and which may also explain the poor diffracting power of these crystals. Data were scaled using *HKL*-2000 and a summary of processing statistics is shown in Table 1.

3. Results and discussion

In addition to SeMet-derivatized protein crystals, we also grew crystals of native protein in 20% polyethylene glycol 10K and 0.1 *M* MES pH 6.5; a data set was collected from one of these crystals on beamline BM14 at the ESRF, France. These crystals diffracted to 4.0 Å resolution at best. The data were processed with *HKL*-2000 (Otwinowski & Minor, 1997) and interestingly these native crystals belonged to space group $P4_22_12$, with unit-cell parameters $a = 108.59$, $b = 108.59$, $c = 271.72$ Å ($\alpha = \beta = \varphi = 90°$). Previously, native crystals of PaGDH grown at the University of Sheffield, England diffracted to a maximum resolution of 4.0 Å (Waugh, 1994) and belonged to space group *H*32, with unit-cell parameters $a = b = 155.1$, $c = 168.7$ Å. Interestingly, these native crystals were also grown in the presence of ammonium sulfate (2 *M*) but with the addition of tetracyanoplatinate at pH 6. Given the history of poorly diffracting native crystals, we focused our work on SeMet-derivatized protein and diffraction data for further stages of structure determination.

Assuming the presence of two molecules in the asymmetric unit of the SeMet-derivatized crystals, the calculated Matthews coefficient was 3.84 $Å^3\,Da^{-1}$, indicating a solvent content of 67.97%. We attempted to incorporate the anomalous signal from the 19 Se atoms following a single-wavelength anomalous diffraction (SAD) experiment. Despite the anomalous signal only being reliable to 6.5 Å, 19 of 22 heavy-atom positions were found. However, after density modification and phase extension the secondary-structure elements were not apparent in the resulting electron-density maps.

In parallel, the glutamate dehydrogenase from *Thermococcus profundus* (Nakasako *et al.*, 2001; PDB code 1euz) was used as a search model for molecular replacement using the program *Phaser* (McCoy *et al.*, 2007). This enzyme shares 46% sequence identity with PaGDH. A solution comprising two molecules was found with rotation-function and translation-function *Z* scores of 7.6 and 16.1, respectively, and a log-likelihood gain (LLG) of 206. The current R and $R_{\rm free}$ values are 44.9% and 55.5%, respectively. We are attempting model building and refinement of the structure by iterative cycles of building in *Coot* (Emsley & Cowtan, 2004) and refinement with *REFMAC*5 (Murshudov *et al.*, 1997). Inspection of the maps reveals that the 19 Se positions are consistent with the locations of methionine residues in PaGDH. Therefore, we are attempting to incorporate the anomalous data from the 19 Se atoms to reduce model bias. In the meantime, crystals are being optimized to improve diffraction quality and it is expected that a more intense beamline will help in improving the data sufficiently to facilitate refinement of the structure.

Table 1
Summary of X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray wavelength (Å)	0.978
Space group	*H*32
Unit-cell parameters (Å)	$a = 154.82$, $b = 154.82$, $c = 309.64$
Resolution (Å)	3.5 (3.63–3.5)
Total No. of reflections	234767
No. of unique reflections	18437
Completeness (%)	96 (88.1)
$\langle I/\sigma(I)\rangle$	13.4 (1.75)
$R_{\rm merge}$† (%)	16.8 (67.6)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

It has been nearly 20 years since PaGDH was cloned (Snedecor *et al.*, 1991); this was soon followed by the preparation of initial crystals in the laboratory of David Rice at the University of Sheffield (Waugh, 1994). However, the structure has not yet been determined. In this study, we also encountered difficulties in growing diffraction-quality crystals for structure determination using the native enzyme. In an attempt to overcome this problem, SeMet-derivatized protein was purified and a significant improvement in diffraction was observed. Improvement in diffraction quality on SeMet substitution has been observed previously (Sulzenbacher *et al.*, 2001), although the effects of Se on lattices are not well characterized. Several methionine residues in PaGDH may mediate intersubunit and lattice contacts that could be affected by derivatization of the protein. However, to complicate the issue further we have observed variations in the space group and diffraction limits of crystals that do not appear to be linked to surface-exposed methionine/SeMet residues (Jagoe, Jackson *et al.*, 2006; Jagoe, Lindsay *et al.*, 2006). It is likely that the mechanisms underlying native crystal *versus* SeMet crystal formation are likely to be diverse and dependent on the nature of the protein and crystal lattice. Given the ease of preparing recombinant protein using minimal media in *E. coli*, SeMet derivatization should be considered routinely to overcome the problem of poorly diffracting native crystals.

Preliminary analysis of the PaGDH structure reveals a hexameric assembly formed by three copies of the asymmetric dimer. The hexamer is arranged in a conventional manner compared with the bacterial and mammalian enzymes. However, the current data are insufficient to provide details of side chains and therefore refinement of the structure is proving to be a challenge. It is expected that a very modest improvement in data quality will enable refinement and provide a deeper understanding of the structural basis of the various ways of achieving coenzyme specificity in the glutamate dehydrogenase family.

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