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Towards the crystal structure of glycerol dehydrogenase from *Thermotoga maritima*

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The NAD^+-dependent glycerol dehydrogenase (EC 1.1.1.6) from the extremely thermophilic bacterium *Thermotoga maritima* has been crystallized in the presence of glycerol by the hanging-drop vapour-diffusion method using 2-methyl-2,4-pentanediol (MPD) as the precipitating agent. Crystals of the enzyme complexed with NAD^+ have also been obtained. The crystals belong to the tetragonal system with space group $I422$ and unit-cell parameters $a = 105.3$, $c = 134.5$ Å. They diffract to a maximum resolution of 1.4 Å using synchrotron radiation ($\lambda = 0.838$ Å). Crystals of the enzyme–NAD^+ complex diffract to 2.5 Å resolution using in-house Cu $K\alpha$ radiation.

1. Introduction

T. maritima is a rod-shaped anaerobic eubacterium that was originally isolated from geothermally heated marine sediments at Vulcano, Italy. It can thrive at temperatures of up to 363 K (Huber *et al.*, 1986) and is one of the most thermophilic bacteria currently known. The organism is of great evolutionary significance; small-subunit ribosomal RNA (ssU rRNA) phylogeny has placed it as one of the deepest and most slowly evolving lineages within the bacteria.

Glycerol dehydrogenase is a cytoplasmic protein and, although the bacterium is anaerobic, it is tolerant towards oxygen. The enzyme converts glycerol to dihydroxyacetone (Fig. 1), which is subsequently phosphorylated to dihydroxyacetone phosphate before entering the glycolytic pathway. The enzyme contains zinc and is a homotetramer in solution. Each subunit has a molecular weight of approximately 42 kDa and consists of 364 amino-acid residues.

Glycerol dehydrogenases have been isolated from a number of different sources including mammalian (Kormann *et al.*, 1972) and bacterial species (Lin, 1976). An alignment of the primary sequence of glycerol dehydrogenase from *T. maritima* with those from other organisms for which sequences are available was performed using the program *CLUSTAL* (Higgins *et al.*, 1996) in order to determine the number of conserved residues. 122 of the 364 amino-acid residues are identically conserved among the glycerol dehydrogenases aligned, but there is no representative crystal structure for this class of enzyme in the Protein Data Bank (Bernstein *et al.*, 1978) at present. The crystal structure of this metalloenzyme will therefore provide insight into its substrate-binding and coenzyme-binding properties as well as the role of metal ions in its catalytic activity. The crystal structure determination will also provide insights into the thermostable characteristics of the enzyme and will enable comparisons to be made with its mesophilic counterparts.

2. Materials and methods

2.1. Protein purification

Glycerol dehydrogenase was purified from *T. maritima* cells grown at 353 K using glucose as the carbon source. The successive columns used for liquid chromatography were DEAE fast flow (5 × 12 cm), hydroxyapatite (5 × 10 cm), phenyl-Sepharose (3.5 × 10 cm) and gel-filtration Superdex 200 (6 × 60 cm). All chromatography steps were carried out at room temperature using 50 m*M* Tris pH 8.0 as the buffer. The purity of the protein was judged by SDS gel electrophoresis.

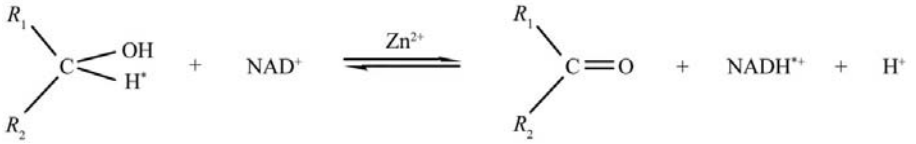

Figure 1
Reaction catalyzed by glycerol dehydrogenase. $R_1 = R_2 = CH_2OH$ in glycerol dehydrogenase.

2.2. Crystallization

The enzyme was stored at a concentration of 36 mg ml^{-1} in 50 m*M* Tris buffer pH 8.0 and 10% glycerol. Crystallization experiments were performed by the hanging-drop vapour-diffusion method with the protein at a concentration of 12 mg ml^{-1}. Hampton Crystal Screens I and II (Cudney *et al.*, 1994) were tried by mixing 2 µl of the protein solution with 2 µl of the reservoir solution. Well formed tetragonal crystals (Fig. 2) were obtained from a reservoir containing 30% 2-methyl-2,4-pentanediol (MPD) in 0.2 *M* sodium citrate buffer pH 5.6 at 295 K.

Crystallization of the complex of glycerol dehydrogenase with NAD^+ was performed using the crystallization buffer above including 2 µl of a 10 m*M* NAD^+ solution in the drop. The crystal morphology was very similar to the uncomplexed crystals.

2.3. Activity assay

Glycerol dehydrogenase activity was assayed at 353 K and under anaerobic conditions by measuring the glycerol-dependent reduction of NAD^+ at 340 nm. The crystals were first transferred to a solution containing 30% 2-methyl-2,4-pentanediol (MPD) in 0.2 *M* sodium citrate buffer pH 5.6 (crystallization buffer) and centrifuged. The supernatant solution was carefully removed and the crystals were dissolved in distilled water for analysis. The concentration of the above solution was determined by the Bradford method (Marshall & Williams, 1992) using bovine serum albumin as the standard. The reaction mixture (2 ml) contained 100 m*M* *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) (EPPS) pH 8.6, 5%(*w*/*v*) glycerol and 0.5 m*M* NAD. The reaction was initiated by addition of the enzyme solution prepared by dissolving the crystals as stated above. The first batch of enzyme crystals had an activity of 96 units per milligram of enzyme, where 1 unit equals 1 µmol of NAD reduced per minute.

2.4. Metal assay

A complete metal analysis (20 elements, including zinc and iron) was performed by plasma-emission spectroscopy using the Thermo Jarrell-Ash 965 Inductively Coupled Argon Plasma (ICAP) at the Chemical Analysis Laboratory, University of Georgia. The aqueous sample was prepared by washing the crystals in a buffer free of metals and dissolving them in distilled water. The crystals showed the presence of only zinc (0.3 Zn atoms per subunit). This suggests that each subunit contains one or more zinc ions but that most of the metal is lost during purification. Alternatively, one zinc ion could be bound to the tetrameric form of the enzyme.

Table 1
Data-collection parameters.

	Native	Complex with NAD	Pt derivative
Crystal dimensions (mm)	0.25 × 0.25 × 0.4	0.2 × 0.2 × 0.3	0.25 × 0.25 × 0.4
Crystal-to-detector distance (mm)	250.0	190.00	320.0
Maximum data resolution (Å)	1.4	2.53	2.35
Oscillation range (°)	0.5	0.5	0.5
Temperature (K)	100	100	100
Exposure time	Dose = 60 000 counts	15 min	Dose = 40 000 counts
Wavelength (Å)	0.838	1.54	1.0
Total crystal rotation (°)	90	90	60
Unit-cell parameters (Å)			
$a = b$	105.3	104.7	105.4
c	134.5	135.0	134.8
Space group	*I*422	*I*422	*I*422

Table 2
Data-processing statistics.

Values in parentheses are for the highest resolution shell.

Data set	Resolution (Å)	No.of observations/ unique reflections	Completeness (%)	R_{sym}† (%)	$I/\sigma(I)$
Native	15–1.4 (1.45–1.40)	1894206/74223	97.9 (84.7)	3.6	46.3 (5.3)
Derivative K_2PtCl_4	15–2.35 (2.43–2.35)	267360/15907	81.6 (85.1)	5.2	22.1 (3.9)
Native + NAD	20–2.53 (2.57–2.53)	275144/15061	98.3 (89.7)	8.7	15.0 (2.43)

† $R_{sym} = (\sum |I - \langle I \rangle|)/\sum(I)$, where I is the observed intensity and $\langle I \rangle$ is the average intensity of symmetry-related reflections.

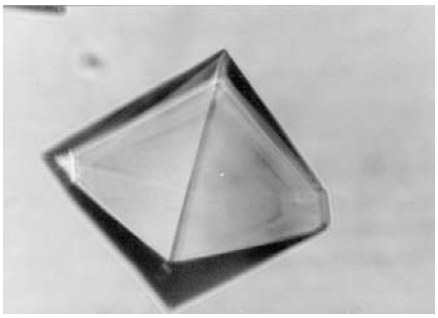

Figure 2
Tetragonal crystal of glycerol dehydrogenase. Dimensions of the crystal are 0.25 × 0.25 × 0.4 mm.

2.5. Data collection and preliminary analysis

Data were collected on the XW7A beamline (λ = 0.838 Å) of the DORIS storage ring at the EMBL outstation at the Deutsches Elektronen Synchrotron (DESY), Hamburg, Germany. A single crystal exposed to a cold nitrogen stream was used to record the entire data set using a MAR 345 imaging-plate detector. The MPD from the well solution provided sufficient cryoprotection for this experiment. The crystal diffracted to a resolution of 1.4 Å. A diffraction pattern of the crystal is shown in Fig. 3.

90° of data were collected by setting the collection mode of the detector to 'dose mode' for 60 000 counts. The 'dose mode' takes care of the natural decay of the primary beam intensity at synchrotron sources, ensuring that the integrated intensity of the primary beam is kept constant for each data-collection frame. Because many of the low-resolution reflections were overloaded and reached the maximum dynamic range of the detector, the intensity of the

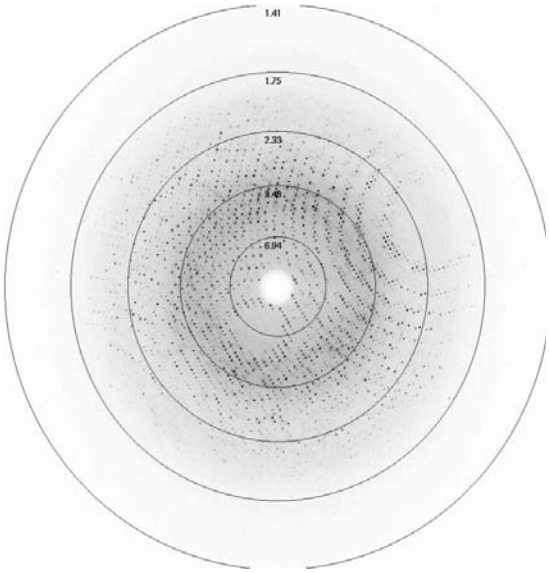

Figure 3
A 0.5° oscillation diffraction image of the glycerol dehydrogenase crystal collected using a MAR 345 imaging-plate detector. The outer edge of the plate is at 1.4 Å resolution.

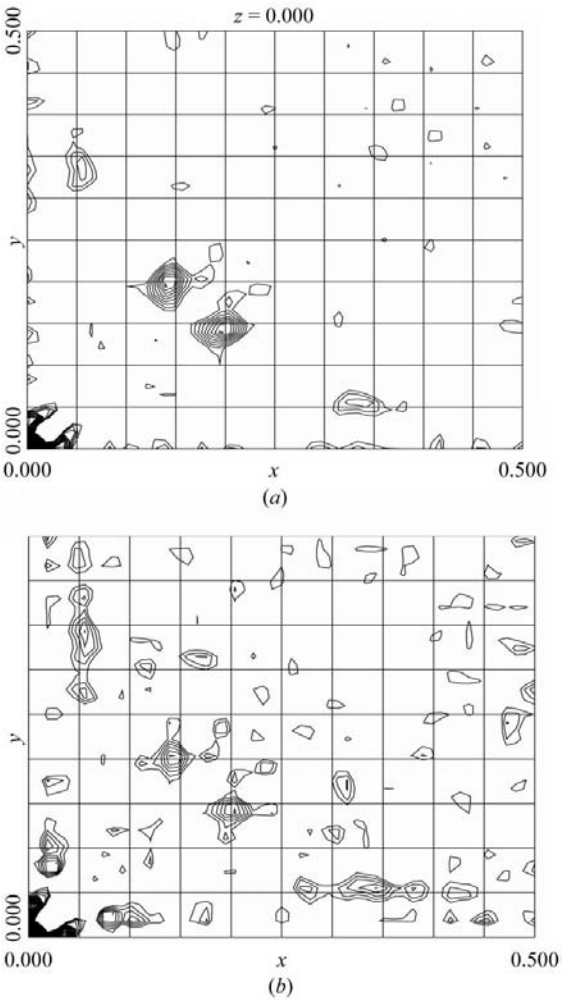

Figure 4
(*a*) Isomorphous and (*b*) anomalous difference Patterson maps showing the 'Pt' peak in the $w = 0$ Harker section. The maps are contoured with a cutoff and increment of 1σ.

beam was attenuated by a factor of ten and another fast sweep of data collection in the low-resolution range (15–4.0 Å) using a crystal oscillation of 0.5° was measured.

All diffraction data were autoindexed and integrated using the program *DENZO* (Otwinowski & Minor, 1997). Scaling and merging of the data was performed with the program *SCALEPACK* (Otwinowski & Minor, 1997). Systematic absences in the data ($h + k + l = 2n + 1$) indicated space group *I*422, with unit-cell parameters $a = b = 105.3$, $c = 134.5$ Å. Data-collection statistics are shown in Table 1. Given the subunit molecular weight of 42 kDa, the asymmetric unit contains one subunit molecule with a Matthews coefficient (Matthews, 1968) of 2.34 $Å^3\ Da^{-1}$ and a solvent content of 47%.

Data for the crystal complexed with NAD^+ were recorded on a MAR Research 300 image-plate scanner (Cu $K\alpha$ radiation) at the X-ray Crystallographic facility at the University of Georgia. The crystal diffracted to 2.5 Å and had the same space group, *I*422, with unit-cell parameters $a = b = 104.7$, c = 135.0 Å. Data-collection and processing statistics are shown in Tables 1 and 2. Despite the fact that both glycerol and NAD are present in the crystals, the conversion of glycerol to dihydroxyacetone is too slow to be significant at room temperature and does not present a problem in the diffraction measurements.

For structure solution, isomorphous heavy-atom derivative crystals were prepared by soaking the native crystals in a solution of 0.5 m*M* K_2PtCl_4 for 90 min at room temperature. Data were collected on the XW7B beamline ($\lambda = 1.00$ Å) at the EMBL Outstation, Hamburg, Germany. The derivative crystals diffracted to 2.35 Å resolution.

Isomorphous difference and anomalous difference Patterson maps were computed using the program *PHASES* (Furey & Swaminathan, 1997) as shown in Fig. 4. The difference Patterson maps show strong peaks for the Pt position. The structure solution is currently in progress using both the isomorphous and anomalous differences of the Pt atom for phase determination.

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