Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and quaternary structure analysis of a glycerol dehydrogenase S305C mutant from *Bacillus stearothermophilus*

Jacky Burke,[a] Sergey N. Ruzheinikov,[a] Sveta Sedelnikova,[a] Patrick J. Baker,[a] David Holmes,[b] Nicola M. Muir,[c] Michael G. Gore[c] and David W. Rice[a]*

[a]Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, England, [b]EM Unit, School of Biological Sciences, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, England, and [c]Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton, England

Correspondence e-mail: d.rice@sheffield.ac.uk

Received 31 May 2000
Accepted 23 October 2000

Bacillus stearothermophilus glycerol dehydrogenase (GlyDH) is a 39.5 kDa molecular weight metalloenzyme which catalyzes the oxidation of glycerol to dihydroxyacetone with the concomitant reduction of NAD^+ to NADH. Despite its classification as a member of the 'iron-containing' polyol dehydrogenase family, studies on recombinant *B. stearothermophilus* GlyDH have shown this enzyme to be Zn^{2+}-dependent. Crystals of a S305C GlyDH mutant were obtained by the hanging-drop vapour-diffusion method, using ammonium sulfate and PEG 400 as precipitating agents, in the presence and absence of NAD^+. The crystals belong to space group *I*422, with approximate unit-cell parameters $a = b = 105$, $c = 149$ Å and one subunit in the asymmetric unit, corresponding to a packing density of 2.6 $Å^3$ Da^{-1}. The crystals diffract X-rays to at least 1.8 Å resolution on a synchrotron-radiation source. Determination of the structure will provide insights into the key determinations of catalytic activity of this class of enzymes, for which no structures are currently available.

1. Introduction

Two pathways exist in bacteria for the dissimilation of glycerol. One pathway involves the use of an $NADP^+$-linked glycerol dehydrogenase, which converts glycerol to glyceraldehyde (Viswanath-Reddy *et al.*, 1978). The second pathway involves the oxidation of glycerol to dihydroxyacetone with the concomitant reduction of NAD^+ to NADH (May & Sloan, 1981). In *B. stearothermophilus*, the latter reaction is catalysed by glycerol dehydrogenase (GlyDH; glycerol:NAD^+ 2-oxidoreductase; E.C. 1.1.1.6; Spencer *et al.*, 1989). Studies on recombinant *B. stearothermophilus* GlyDH have shown this enzyme to be Zn^{2+}-dependent (Paine *et al.*, 1993).

The gene for *B. stearothermophilus* glycerol dehydrogenase has been cloned (Mallinder *et al.*, 1992) and analysis of the derived amino-acid sequence has shown that the core of this enzyme has a limited sequence homology to a number of alcohol dehydrogenases (ADHs) which belong to the third ADH family, the so-called 'iron-containing' ADHs, for which no representative three-dimensional structure is currently available.

2. Materials and methods

The gene for GlyDH has been cloned in the expression vector pKK233-2 under the control of the IPTG-inducible *tac* promotor together with an ampicillin-resistance gene and has been transformed into the *Escherichia coli* cell line JM103. Previous efforts to crystallize wild-type GlyDH yielded a number of different crystal forms with diffraction only to 3.0 Å (Wilkinson *et al.*, 1995). The S305C mutant was designed as one of a series of mutations to assist in the determination of the structure of the enzyme by creating novel heavy-atom sites by cysteine mutagenesis. Biochemical studies show that the S305C mutation leads to little change in the catalytic properties of enzyme (data not shown).

The *E. coli* JM103 cells harbouring the plasmid containing the GlyDH S305C gene were grown at 310 K, with shaking at 200 rev min^{-1}, in the presence of ampicillin until an OD_{600} of between 0.6 and 0.9 was reached. The cells were then induced with 1 m*M* IPTG and grown for a further 16 h; the cells were then harvested by centrifugation and frozen.

To purify GlyDH, 3 g of cell paste was defrosted and suspended in buffer *A* (40 m*M* Tris–HCl pH 8.5, 0.1 m*M* $ZnCl_2$). The cells were disrupted by ultrasonication for 3×20 s at 16 µm amplitude and cell debris was removed by centrifugation at 42 000*g* for 15 min. The crude extract typically contained a total of 150–200 mg protein, estimated by the method of Bradford (1976) using the Bio-Rad Protein Assay reagent. Ammonium sulfate solution (4 *M*) was added to the crude extract to a final concentration of 1.9 *M* and the precipitated proteins were removed by centrifugation. The supernatant fraction was then applied to a 30 ml butyl-Toyopearl 650S

column equilibrated with 1.8 *M* ammonium sulfate in buffer *A*. The protein was eluted from this column with a 250 ml reverse concentration gradient of ammonium sulfate from 1.75 to 0 *M* in buffer *A*. Fractions with the highest protein concentration were combined and concentrated on a Viva-Spin concentrator to reduce the volume to 1–2 ml. The sample was then applied to a 1.6 × 60 cm Hi-Load Superdex-200 (Pharmacia) column equilibrated with buffer *A*. S305C glycerol dehydrogenase was eluted from the column with an apparent molecular mass of 305 kDa. Analysis of the wild-type protein under equivalent conditions gave an apparent molecular mass between 400 and 630 kDa, indicating a tendency of wild-type GlyDH to form larger species, at least under these buffer conditions.

The peak fractions of S305C GlyDH were combined and concentrated on a Viva-Spin concentrator to a concentration of 15 mg ml^{-1} and this sample was used for crystallization trials. The typical yield was about 10 mg of protein from a total of 100 mg of protein in the crude extract. The protein purity, estimated by PAGE (Nu-PAGE 4–12% bis-tris pre-cast gel, MES SDS running buffer, NOVEX), was in the range 90–95%.

Scanning transmission electron microscopy (STEM) on a Jeol 1200EX with ASID10 scanning attachment and modified for external scan control and image acquisition (Holmes, 1995) was used to measure the particle mass of GlyDH. The enzyme was adsorbed from buffer onto carbon-filmed grids, washed with ultrapure water, drained and air-dried. Tobacco mosaic virus was used as a standard of mass per unit length (131 kDa nm^{-1}) and a diffraction grating replica (2160 lines mm^{-1}) was used for magnification calibration. STEM images were acquired with an accelerating voltage of 120 kV and with an electron dose on the specimen of about 300 e nm^{-2}, incurring a negligible beam-induced mass loss of 2–3%.

Crystals were grown using the standard hanging-drop vapour-diffusion technique by mixing 4 µl of the protein solution (15 mg ml^{-1}) with 4 µl of the precipitant in the presence (10 m*M*) and absence of NAD$^+$ and equilibrating over the precipitant at 290 K. Trigonal bipyramid-like crystals up to 0.3 × 0.6 × 0.6 mm in size appeared in the presence and absence of cofactor (see Fig. 1) within one week over a wide range of precipitant concentration (0.5–2.0 *M* ammonium sulfate, 2–6% PEG 400 and 20 µ*M* $ZnCl_2$).

X-ray diffraction data from crystals grown in the presence of NAD$^+$ were collected at room temperature to 1.7 Å as 1° rotation frames on a Quantum Q4 CCD detector on station 14.2 at the SRS Daresbury Laboratory with a wavelength of 0.979 Å. X-ray diffraction data from crystals of the free enzyme were collected at room temperature to 1.8 Å as 1° rotation frames on the same detector. The data were processed using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997).

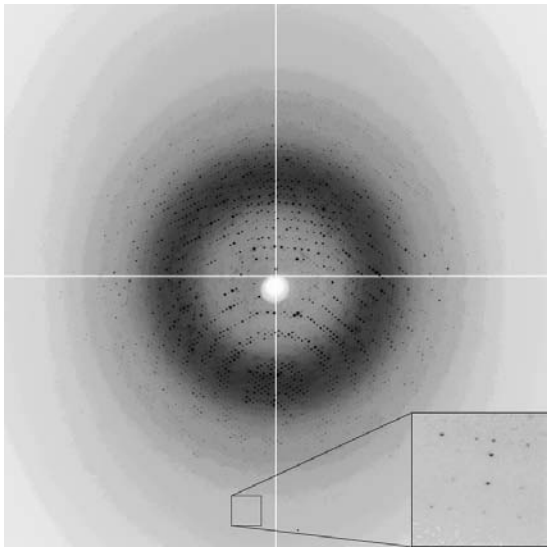

Figure 1
A representative 1° oscillation image of data collected from the GlyDH–NAD$^+$ complex crystal on a Quantum Q4 CCD detector on station 14.2 at the SRS Daresbury Laboratory. The middle of the selected rectangle corresponds to a resolution of 1.7 Å.

3. Results and discussion

The crystals of the S305C mutant of GlyDH were quite stable to X-ray radiation and data collection was therefore performed at room temperature. Analysis of a preliminary data set collected on the crystals grown in the presence of NAD$^+$ with the autoindexing routine in *DENZO* (Otwinowski & Minor, 1997) is consistent with a body-centered tetragonal crystal system, class 422, with unit-cell parameters $a = b = 104.7$, $c = 148.5$ Å. The unit-cell parameters for the free enzyme are closely related ($a = b = 105.0$, $c = 148.2$ Å). Both data sets can also be indexed as a face-centered orthorhombic system (*F*222), which indicates that the crystals belong to the space group *I*422.

For the crystal of the GlyDH–NAD$^+$ complex, significant reflections were observed to 1.7 Å resolution (Fig. 1), with the average $I/\sigma(I)$ value being 19.9 for all reflections and 2.7 in the highest resolution shell (1.74–1.70 Å). A total of 196 167 measurements were made of 42 016 independent reflections. Data processing gave an $R_{\rm merge}$ of 0.047 for intensities (0.376 in the resolution shell 1.74–1.70 Å) and this data set is 92.1% complete (78.6% completeness in the highest resolution shell).

For the crystal of the free enzyme, significant reflections were observed to 1.8 Å resolution (Fig. 1), with the average $I/\sigma(I)$ value being 13.6 for all reflections and 2.7 in the highest resolution shell (1.84–1.80 Å). A total of 194 595 measurements were made of 34 428 independent reflections. Data processing gave an $R_{\rm merge}$ of 0.064 for intensities (0.415 in the resolution shell 1.84–1.80 Å) and this data set is 89.6% complete (83.4% completeness in the highest resolution shell).

Consideration of possible values for $V_{\rm M}$ together with the data from gel filtration (305 kDa) and STEM mass analysis (391 kDa) suggest a quaternary structure based on an octamer in 42 symmetry. Taking the subunit molecular weight to be 39.6 kDa, this would suggest that the crystal contains a monomer in the asymmetric unit, with a $V_{\rm M}$ of 2.6 Å^3 Da^{-1}, which is within the range given by Matthews (1977). These data are in contrast to earlier studies which indicated a tetrameric structure for this enzyme isolated from a related strain of *B. stearothermophilus* (Spencer *et al.*, 1989).

Whilst the crystallization conditions reported here are distinct from those used in the earlier studies, preliminary attempts to reproduce the current crystallization conditions with the wild-type enzyme produced inferior crystals, which again only diffracted to 3.0 Å. Thus, it would appear that the mutation (S305C) that we have introduced is beneficial to the production of high-quality crystals for X-ray analysis.

Our efforts are currently being directed towards a search for heavy-atom derivatives and the solution of the structure using multiple isomorphous replacement.

We thank Dr Anne Moir for the kind donation of the gene for wild-type glycerol dehydrogenase and the support staff at the Synchrotron Radiation Source at the CCLRC Daresbury Laboratory for assistance with station alignment. This work was supported by the New Energy and Industrial Development Organization, the EU, the

Wellcome Trust and the BBSRC. The Krebs Institute is a designated BBSRC Biomolecular Science Centre and a member of the North of England Structural Biology Centre.

References

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Holmes, D. F. (1995). *Biochem. Soc. Trans.* **23**, 720–725.
Mallinder, P. R., Pritchard, A. & Moir, A. (1992). *Gene*, **110**, 9–16.
Matthews, B. W. (1977). *X-ray Structure of Proteins*, edited by H. Neurath & R. L. Hill, 3rd ed., Vol. 3, pp. 468–477. New York: Academic Press.
May, J. W. & Sloan, J. (1981). *J. Gen. Microbiol.* **123**, 183–185.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Paine, T. J., Perry, N., Popplewell, A. G., Gore, M. G. & Atkinson, T. (1993). *Biochem. Biophys. Acta*, **1202**(2), 233–243.
Spencer, P., Bown, K. J., Scawen, M. D., Atkinson, T. & Gore, M. G. (1989). *Biochem. Biophys. Acta*, **994**, 270–279.
Viswanath-Reddy, M., Pyle, J. E. & Branch Howe, H. (1978). *J. Gen. Microbiol.* **107**, 289–296.
Wilkinson, K. W., Baker, P. J., Rice, D. W. & Stillman, T. J. (1995). *Acta Cryst.* D**51**, 830–832.