Crystallization of glycerol dehydrogenase from *Bacillus stearothermophilus*. By KAY W. WILKINSON, PATRICK J. BAKER, DAVID W. RICE and TIMOTHY J. STILLMAN, *Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, PO Box 594, University of Sheffield, Sheffield S10 2UH, England*, MICHAEL G. GORE and OLIVER KRAUSS, *Department of Biochemisty, Institute of Biomolecular Sciences, University of Southampton, Southampton, England*, and TONY ATKINSON, *Microbial Technology Laboratory, PHLS Centre for Applied Microbiology Research, Porton, Salisbury, England*

Abstract

The NAD$^+$-linked glycerol dehydrogenase from *Bacillus stearothermophilus* is a member of the so-called 'iron-containing' class of polyol dehydrogenases. This enzyme has been crystallized in three different forms in the presence of a range of divalent cations and glycerol or NAD$^+$ using the hanging-drop method of vapour diffusion with ammonium sulfate as the precipitant. X-ray photographs have established that the crystals grown in the presence of zinc and glycerol (form *A*) most likely belong to space group $I4_1 22$ with cell parameters $a = b = 102$ and $c = 728$ Å. The crystals grown with zinc and NAD$^+$ (form *B*) belong to the tetragonal system and probably belong to the space group $P4_2 1 2$ with cell parameters $a = b = 150$ and $c = 220$ Å. The crystals grown with lead and glycerol (form *C*) belong to a primitive orthorhombic system with cell parameters $a = 127$, $b = 178$ and $c = 173$ Å. Experiments using the synchrotron radiation source at the DRAL Daresbury laboratory have shown all three crystal types diffract to at least 3 Å resolution. Elucidation of the three-dimensional structure of this enzyme will provide a structural framework for this class of polyol dehydrogenases, which are not represented in the database at present, and enable comparisons to be made with enzymes belonging to the other classes.

Introduction

Glycerol dehydrogenases have now been isolated from a number of different sources including mammalian (Moore, 1959; Kormann, Hurst & Flynn, 1972) and bacterial species (Scharshmidt, Pfleiderer, Metz & Brummer, 1983; Burton & Kaplan, 1953; Lin & Magasanik, 1960). In bacteria, there are two oxidative pathways for glycerol, one of which uses an NADP$^+$-linked enzyme to oxidize glycerol to glyceraldehyde (Viswanath-Reddy, Pyle & Branch Howe, 1978) whilst the other uses an NAD$^+$-linked enzyme to oxidize glycerol to dihydroxyacetone which is subsequently phosphorylated to dihydroxyacetone phosphate before entering the glycolytic pathway (May & Sloan, 1981). The glycerol dehydrogenase from the thermophile *Bacillus stearothermophilus* (E.C. 1.1.1.6) has been shown to belong to the family of NAD$^+$-linked enzymes which use the latter pathway (Spencer, Bown, Scawen, Atkinson & Gore, 1989).

Analysis of the amino-acid sequence data which have been accumulated on a range of alcohol (ADH) and polyol dehydrogenases has shown that these enzymes can be grouped into three distinct protein families. These include a family of zinc-containing 'long-chain' ADH's, a family of insect-type or 'short-chain' ADH's and finally a family of 'iron-containing' ADH's (Bairoch, 1992; Jörnvall, Persson & Jeffery, 1987). Information on the three-dimensional structure of the enzymes belonging to the class of 'long-chain' ADH's has been provided by X-ray analysis of crystals of the tetrameric horse liver alcohol dehydrogenase (Eklund *et al.*, 1976) and the dimeric human $\beta_1\beta_1$ alcohol dehydrogenase (Hurley, Bosron, Hamilton & Amzel, 1991). In these enzymes each subunit folds into a two-domain structure, forming the coenzyme- and substrate-binding domains, with the former resembling the classical coenzyme-binding domain (Rossmann, Moras & Olsen, 1974).

The determination of the three-dimensional structure of $3\alpha,20\beta$-hydroxysteroid dehydrogenase (Ghosh, Weeks, Wawrzak, Duax & Erman, 1994) and rat liver dihydropteridine reductase (Varughese, Skinner, Whiteley, Mathews & Xuong, 1992) has given an insight into the structure–function relationships within the family of 'short-chain' ADH's. These enzymes contain a nucleotide-binding domain with strong structural similarities with the classic dinucleotide-binding fold. However unlike the 'long-chain' ADH's each subunit folds into a single domain which resembles the nucleotide-binding fold. The substrate-binding site is made up from loops connecting secondary-structural elements in the nucleotide-binding domain.

The gene for *B. stearothermophilus* glycerol dehydrogenase has been cloned (Mallinder, Pritchard & Moir, 1992) and analysis of the amino-acid sequence deduced has shown that the protein has a molecular weight of 42 000 Da with limited sequence homology to a number of ADH's which belong to the third ADH family. This family, the so-called 'iron-containing' ADH's, has no representative three-dimensional structure currently available. The family is thought to include *Clostridium acetobutylicum* butanol dehydrogenases (Walter, Bennet & Papoutsakis, 1992), ADH1 and acetaldehyde dehydrogenase, *Zymomonas mobilis* ADH2 (Conway & Ingram, 1989), *Saccharomyces cerevisiae* ADH4 (Williamson & Paquin, 1987), *Escherichia coli* 1,2-propanediol dehydrogenase (Chen, Lu & Lin, 1989) and acetaldehyde dehydrogenase (Goodlove, Cunningham, Parker & Clark, 1989), two methanol:N,N'-dimethyl-4-nitrosoaniline oxidoreductases from the methylotrophic bacteria *Amycolatopsis methancolica* and *Mycobacterium gastri* (Bystrykh *et al.*, 1993), methanol dehydrogenase from *Bacillus methanolicus* (De Vries, Arfman, Terpstra & Dijkhuizen, 1992), an oxygen-labile alcohol dehydrogenase from *Desulfovibrio gigas* (Hensgen, Vonck, van Beeumen, van Bruggen & Hansen, 1993), 4-hydroxybutyrate dehydrogenase from *Clostridium kluyveri* and glycerol dehydrogenase from *E. coli* (Blattner, Burland, Plunkett, Sofia & Daniels, 1993). Alignment of the *B. stearothermophilus* glycerol dehydrogenase with those of the other members of the 'iron-containing' family of dehydrogenases for which sequences are available shows that there are 15 identically conserved amino acids. An additional 22 positions can be identified where six of the eight aligned sequences have identical residues. *B. stearothermophilus* glycerol dehydrogenase shares 47.6% identity with the

equivalent enzyme from *E. coli* and with other members of the family the sequence identity is the highest with the *Z. mobilis* ADH2 (16.3% identity). Previously this group of enzymes was referred to as a family of iron-containing alcohol dehydrogenases, however, studies on recombinant *B. stearothermophilus* glycerol dehydrogenase (Paine, Perry, Popplewell, Gore & Atkinson, 1993) have shown that this enzyme is zinc dependent as is the yeast ADH4 (Drewke & Ciriacy, 1988). Furthermore the *B. methancolius* methanol dehydrogenase contains zinc and magnesium (De Vries *et al.*, 1992) and thus the classification of this family needs to be modified to reflect these data.

The quaternary structure of the *B. methanolicus* C1 methanol dehydrogenase and that of the two methanol:N,N'dimethyl-4-nitrosoaniline oxidoreductases has been studied using electron microscopy. Image processing showed similar structures for all three enzymes and particles with an approximate diameter of 150 Å and displaying a clear fivefold symmetry could be seen. Particles with rectangular cross section and twofold rotational symmetry with dimensions of 90×150 Å were also visible. On the basis of these images it has, therefore, been concluded that these enzymes are decamers with the ten subunits arranged in two rings of five stacked on top of each other.

Cell culture and protein purification

In order to prepare the enzyme for crystallization *E. coli* JM103 cells harbouring the plasmid containing the glycerol dehydrogenase gene were grown for 9 h at 310 K before the induction of protein expression was initiated by the addition of isopropyl-β-D-thiogalactosidase (IPTG, 1 mM). The cells were then grown for a further 16 h and harvested by centrifugation. The pellet was resuspended in 2 vol of 20 mM potassium phosphate buffer, pH 8, also containing 100 μM Zn^{2+} ions and sonicated for 3 min at full amplitude using an MSE soniprep 150 ultrasonicator. The lysed cells were then incubated for 30 min at 273 K in the presence of DNase 1 (1 mg), soybean trypsin inhibitor (1 mg) and 50 mg phenylmethylsulfonyl fluoride (PMSF). The cell debris was removed from solution by centrifugation and the enzyme was concentrated by precipitation using 45–80% ammonium sulfate. After dialysis against buffer (as above) the enzyme was loaded onto a 3×10 cm Q-Sepharose column equilibrated in the same buffer. Eluant from the column was monitored at 280 nm and tested for activity. The column was washed by the loading buffer for 1 h (1.5 ml min^{-1}) and then a single peak containing pure enzyme (specific activity = 25 IU mg^{-1} at 303 K, IU = 1 mol NADH min^{-1} mg protein^{-1}) was eluted by application of a linear gradient formed between 300 ml each of 20 mM and 400 mM potassium phosphate buffer, pH 8.0 also containing 100 μM Zn^{2+} ions. The enzyme was concentrated by re-precipitation with 80% ammonium sulfate and stored at 277 K.

Crystallization of form *A* crystals

Crystallization was carried out using the hanging-drop method of vapour diffusion by mixing 5 μl of the protein solution with 5 μl of the precipitant and equilibrating the drops over the precipitant at 290 K. Trials with ammonium sulfate in the concentration range of 55–70% saturated in 50 mM Hepes buffer, pH 7 containing 20 μM $ZnCl_2$ (buffer *A*) with a protein concentration of 12 mg^{-1} ml^{-1} and including 0.3 M glycerol resulted in the formation of small pyramidal crystals (form *A*). These crystals had maximum dimensions of $0.2 \times 0.2 \times 0.3$ mm and could be stabilized in 75% saturated

ammonium sulfate with 0.3 M glycerol in buffer *A*. X-ray precession photographs of the crystals and test data collected with a MAR scanner on station 9.5 at the synchrotron radiation source (SRS) at the DRAL Daresbury Laboratory show that the crystals belong to the tetragonal system, point group 422, and *hkl* reflections are systematically absent when $h + k + l = 2n + 1$, identifying the space group as either $I4_122$ or $I422$ with cell dimensions $a = b = 102$ and $c = 728$ Å. In view of the length of the *c* axis, packing considerations would suggest that the crystals belong to the space group $I4_122$. Gel-filtration studies indicate that the molecular weight of glycerol dehydrogenase is approximately 190 kDa and, given the subunit molecular weight of 42 kDa, this has been interpreted as indicating a tetrameric quaternary structure (Spencer, 1989). However, gel-filtration analysis of the decameric enzymes of this class of ADH's indicate molecular weights in the range 260–280 kDa. Since the true molecular weights of these complexes are in the range 400–500 kDa, the former values are either anomalously low or indicate association–disassociation phenomena. Thus, at present, the quaternary structure of glycerol dehydrogenase is uncertain preventing a precise analysis of the contents of the unit cell. V_m values within the range commonly observed for proteins (Matthews, 1977) are consistent with three to seven subunits in the asymmetric unit.

Crystallization of form *B* crystals

Crystallization of the form *B* crystals was carried out as above using ammonium sulfate as the precipitant in the range of 65–75% saturation in 50 mM phosphate buffer, pH 7 containing 20 μM $ZnCl_2$ (buffer *B*) with a protein concentration of 12 mg ml^{-1} and including 10 mM NAD$^+$. This resulted in the formation of needle-like crystals of maximum dimensions $0.2 \times 0.2 \times 0.5$ mm which could be stabilized in 80% saturated ammonium sulfate with 10 mM NAD$^+$ in buffer *B*. Analysis of a data set collected with a MAR scanner on station 9.5 at the SRS (see Fig. 1) indicates that the crystals belong to the tetragonal system, point group 422, with cell dimensions $a = b = 150$ and $c = 220$ Å. Examination of these data revealed that the *l* axis is a pure fourfold rotation axis. In contrast, for the *h* axis, reflections with indices $h =$ odd appear to be systematically absent, consistent with the tentative identification of this axis as a twofold screw. These preliminary observations identify the space group as $P42_12$. Considerations of the possible values of V_m suggest that this crystal form contains between two and eight subunits in the asymmetric unit.

Crystallization of form *C* crystals

The crystallization trials have also examined the effects of replacing the Zn^{2+} ion in the enzyme with other divalent cations for example Pb^{2+}, Co^{2+} or Cd^{2+}. For these crystallizations, samples of concentrated zinc enzyme were dialysed extensively against 50 mM Hepes buffer containing 10 mM EDTA and 50% ammonium sulfate, in order to remove the enzyme-bound zinc. This was followed by dialysis of the metal-depleted enzyme against 50 mM Hepes buffer containing the different divalent cations at a concentration of 20 μM. Crystallization was carried out as above and trials with ammonium sulfate as the precipitant in the concentration range of 70–90% saturated in 50 mM Hepes buffer, pH 7 containing 20 μM Pb(NO$_3$)$_2$ (buffer *C*) with a protein concentration of 12 mg ml^{-1} and including 0.3 M glycerol resulted in the formation of crystals (form *C*) with a somewhat variable morphology and having maximum dimensions of $0.5 \times 0.3 \times 0.1$ mm. The crystals could be stabilized in 85% saturated ammonium sulfate with 0.3 M

glycerol in buffer C. Smaller crystals with a similar morphology were obtained from equivalent trials in the presence of Cd^{2+} or Co^{2+}. Data collected with a MAR scanner on station 9.5 at the SRS (see Fig. 2) show that the lead-containing form C crystals belong to the orthorhombic system, point group 222, with cell dimensions of $a = 127$, $b = 178$ and $c = 173$ Å. Examination of these data provide clear evidence that the 127 Å axis is a pure twofold rotation, whilst the 173 Å axis appears to be a twofold screw. Reflections along the 178 Å axis were not recorded in this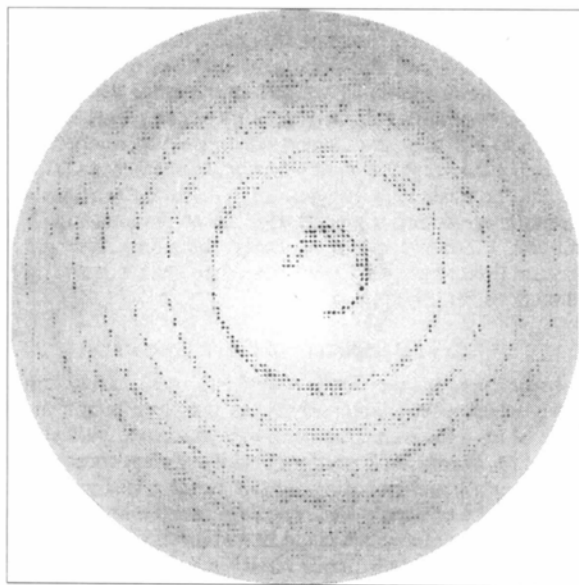 data set and so the space group can be either $P222_1$ or $P2_12_12$. Arguments similar to those presented above would indicate that this crystal form contains between six and 13 subunits in the asymmetric unit, a value which is approximately double that for the other two forms.

All three crystal forms are suitable for structural studies and preliminary efforts are being directed towards the solution of the structure of the form B crystals. The X-ray studies on glycerol dehydrogenase from $B.\ stearothermophilus$ will play a vital role in developing a full understanding of the structure–function relationships in this enzyme and will enable comparisons to be made with the wider enzyme superfamily.

Fig. 1. A 1° oscillation image of a form B crystal taken using the MAR image plate of station 9.5 at the SRS. The resolution edge of the image is 3.5 Å.
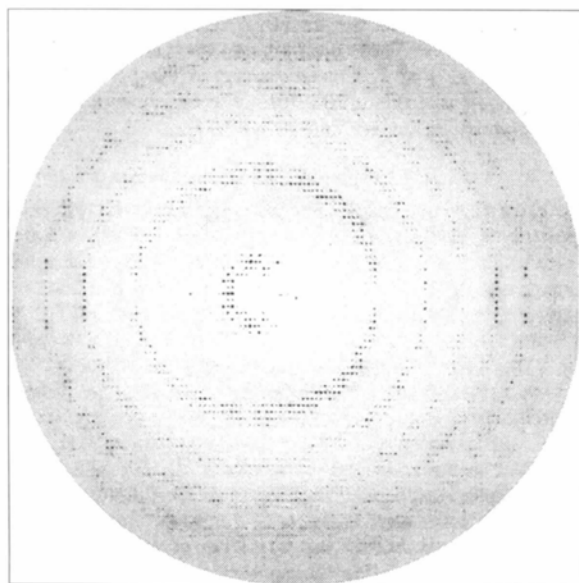

Fig. 2. A 1° oscillation image of a form C crystal taken using the MAR image plate of station 9.5 at the SRS. The resolution edge of the image is 3 Å.

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