Acta Cryst. (1997). D53, 802–804

Crystallization and preliminary X-ray diffraction analysis of the heterotetrameric dihydroorotate dehydrogenase B of *Lactococcus lactis*, a flavoprotein enzyme system consisting of two PyrDB subunits and two iron–sulfur cluster containing PyrK subunits

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(*Received 22 January 1997; accepted 22 April 1997*)

Abstract

Dihydroorotate dehydrogenases are flavin-containing enzymes which catalyze the conversion of (*S*)-dihydroorotate to orotate. Dihydroorotate dehydrogenase B (DHODB) from *Lactococcus lactis* is a heterotetramer containing two subunits of the protein encoded by the *pyrDb* gene (PyrDB) and two subunits of the protein encoded by the *pyrK* gene (PyrK). In addition, DHODB contains two molecules of flavin mononucleotide, two molecules of flavin adenine dinucleotide and two [2Fe–2S] iron–sulfur clusters as tightly bound cofactors. Yellow crystals of this enzyme have been grown using the hanging-drop vapour-diffusion technique from solutions of 2.5 *M* ammonium sulfate and 0.1 *M* sodium acetate, pH 4.6. The crystals have been shown to contain both the PyrDB and the PyrK subunits and fluorescence measurements indicate that the two different subunits interact very closely with each other in the active-site region. Native data sets have been collected to 2.6 Å with a conventional X-ray source and to 2.2 Å using synchrotron radiation. The crystals are rhombohedral, space group *R*32, with corresponding hexagonal unit-cell dimensions $a = b = 202.3$ and $c = 81.0$ Å. The asymmetric unit in the crystal contains one PyrDB subunit and one PyrK subunit, which suggests that the two halves of the heterotetramer are related by a crystallographic twofold axis.

1. Introduction

Dihydroorotate dehydrogenases (DHOD's) are flavin mononucleotide (FMN) containing enzymes. They catalyze the oxidation of (*S*)-dihydroorotate to orotate, the fourth step in the *de novo* biosynthesis of pyrimidine nucleotides. *Lactococcus lactis* is the only organism known to contain two different DHOD's (Andersen, Jansen & Hammer, 1994). Both of these enzymes appear to be biosynthetic in nature, as either is able to complement the lack of dihydroorotate dehydrogenase in a strain of *E. coli* deleted for the *pyrD* gene, and because both of the enzymes must be inactivated by mutation in *L. lactis* in order to impose a pyrimidine requirement on the organism (Andersen, Martinussen & Hammer, 1996).

One of the enzymes, dihydroorotate dehydrogenase A (DHODA), encoded by the *pyrDa* gene, is a dimeric enzyme containing one molecule of FMN per subunit. It is an efficient and stable enzyme that can use various electron acceptors including dichloroindophenol, potassium hexacyanoferrate (III), fumarate and, to some extent, also molecular oxygen to reoxidize the reduced FMN after oxidation of the enzyme substrate (Nielsen, Rowland, Larsen & Jensen, 1996). In contrast, dihydroorotate dehydrogenase B (DHODB) is a heterotetramer formed of two polypeptides encoded by the *pyrDb* gene, *i.e.* the PyrDB subunits, and two polypeptides encoded by the *pyrK* gene, *i.e.* the PyrK subunits (Andersen, Martinussen & Hammer, 1996; Nielsen, Andersen & Jensen, 1996). This enzyme is able to use NAD^+ as an electron acceptor besides the more usual acceptors dichloroindophenol and potassium hexacyanoferrate (III). It contains two molecules of FMN, two molecules of flavin adenine dinucleotide (FAD) and two [2Fe–2S] iron–sulfur clusters as tightly bound cofactors (Nielsen, Andersen & Jensen, 1996). Both types of subunits are needed in the complex to provide an efficient enzyme function both *in vivo* and *in vitro* (Andersen, Martinussen & Hammer, 1996; Nielsen, Andersen & Jensen, 1996). However, the protein encoded by the *pyrDb* gene alone could also be purified as a functional, although inefficient and unstable dimeric DHOD. This contains only FMN as cofactor and was unable to use NAD^+ (Nielsen, Andersen & Jensen, 1996).

The polypeptides encoded by the *pyrDa* and the *pyrDb* genes of *L. lactis* both consist of 311 amino acids but they show only 30% sequence identity. The first of these is 71% identical to the cytosolic DHOD from *Saccharomyces cerevisiae*, whereas the

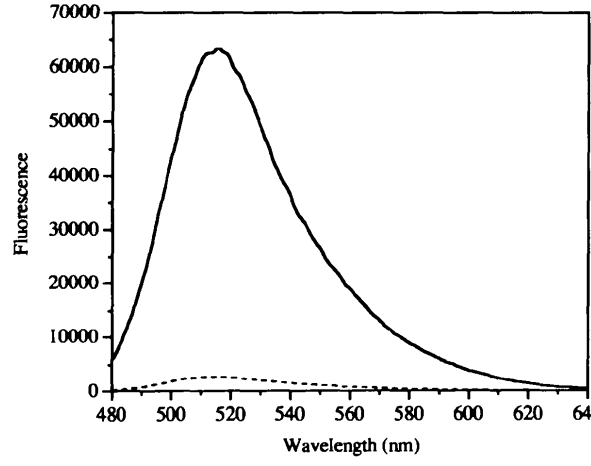

Fig. 1. Fluorescence emission spectra for the dimeric and tetrameric forms of dihydroorotate dehydrogenase B. The proteins were dissolved at concentrations of 0.5 mg ml^{-1} in 50 m*M* sodium phosphate, pH 6.0, containing 10% glycerol. The spectra were recorded with a Perkin Elmer LS-50B luminescence spectrometer with an excitation wavelength of 449 nm, and the fluorescence intensities were corrected to 1 mg of enzyme per ml. Solid line, the dimeric dihydroorotate dehydrogenase; dotted line, the heterotetrameric dihydroorotate dehydrogenase. When normalized relative to the absorption at 449 nm, the fluorescence intensity of the dimeric enzyme was 1.17 times that of FMN while the fluorescence intensity of the tetrameric enzyme was 0.10 times that of FMN.

second closely resembles the enzymes from *Bacillus subtilis* and other Gram-positive bacteria (Nielsen, Rowland, Larsen & Jensen, 1996). Neither of the two *L. lactis pyrK*-encoded polypeptides are closely related to the dimeric membrane bound DHOD of *E. coli* (Larsen & Jensen, 1985) which shows a high level of sequence similarity with the DHOD's of mitochondrial origin. However, both the PyrDB subunit (311 residues) and the PyrK subunit (262 residues) of *L. lactis* DHODB have more than 30% sequence identity with parts of the much larger (about 100 kDa) dihydropyrimidine dehydrogenases found in many organisms. These can reduce all natural pyrimidine bases and the synthetic analogues used in chemotherapy. At present there are no crystal structures known for these enzymes, but they have been reported to contain both flavin groups and iron–sulfur clusters on a single protein chain (Podschun, Cook & Schnackerz, 1990). These similarities suggest that the PyrDB and the PyrK subunits of DHODB may resemble different domains in the dihydropyrimidine dehydrogenases.

Dihydroorotate dehydrogenase A from *L. lactis* has previously been crystallized (Nielsen, Rowland, Larsen & Jensen, 1996) and its crystal structure recently determined (Rowland, Nielsen, Jensen & Larsen, 1997). Here we report the crystallisation and preliminary X-ray diffraction analysis of the heterotetrameric dihydroorotate dehydrogenase B of *L. lactis*. These crystals should facilitate the structure determination of the protein complex, which will allow detailed comparisons to be made between the two *L. lactis* enzymes, and investigation of the interactions between the redox centres and the protein.

2. Results and discussion

2.1. *The protein*

The complex dihydroorotate dehydrogenase B was purified from an extract of *Escherichia coli* that carried the *pyrDb* and *pyrK* genes of *L. lactis* cloned on a multicopy plasmid as described by Nielsen, Andersen & Jensen (1996). The native enzyme is a heterotetramer composed of two PyrDB subunits and two PyrK subunits, and it contains two FMN molecules bound to the PyrDB subunits as well as two FAD molecules and two [2Fe–2S] iron–sulfur clusters, probably bound to the PyrK subunits (Nielsen, Andersen & Jensen, 1996).

When expressed individually in *E. coli*, the PyrDB subunit could be purified as a functional, dimeric dihydroorotate dehydrogenase containing two FMN molecules per dimer, but the protein was an inefficient and unstable catalyst with a half-life of about 40 s at 310 K (Nielsen, Andersen & Jensen, 1996). On the other hand, when the PyrK subunit was expressed alone in *E. coli*, it was either insoluble or it was a soluble, but cofactor-less protein, depending on the growth temperature of the cells (F. S. Nielsen, unpublished observations). In contrast the tetrameric enzyme is a very stable protein that denatured at about 333 K and could be stored at 277 K in 50 m*M* phosphate, pH 6.0, containing 10% glycerol and 0.02% sodium azide for several months without loss of activity.

The dimeric dihydroorotate dehydrogenase, composed of two PyrDB subunits, is strongly fluorescent, while the heterotetramer, composed of two PyrDB and two PyrK subunits, only shows a weak fluorescence when excited in the absorption maximum of FMN (Fig. 1). This shows that the presence of the PyrK subunit in the tetrameric dihydroorotate dehydrogenase strongly affects the immediate surroundings of the FMN moiety bound to the PyrDB subunits, and thus that the two different subunits interact very closely with each other in the active-site region.

2.2. *Crystallization*

An initial search for suitable crystallization conditions was carried out using the hanging-drop vapour-diffusion technique with the standard sparse-matrix crystal-screening solutions (Jancarik & Kim, 1991) from Hampton Research (Crystal Screen and Crystal Screen II). Equal volumes of the crystallization buffer and a protein solution of 16 mg ml^{-1} (in 50 m*M* sodium phosphate, pH 6.0, with 10% glycerol) were used in 4 µl hanging drops at room temperature. After several days, one small triangular prism-shaped crystal appeared in one of the drops containing 2.0 *M* ammonium sulfate and 0.1 *M* sodium acetate at pH 4.6. This crystal was the same orange–brown colour as the purified protein solution. Subsequent variations in the crystallization conditions produced small rod-shaped crystals as well as the triangular prisms. Though the rod-shaped crystals had less sharply defined edges and appeared to be more yellow in colour than the triangular prisms, the X-ray diffraction experiments revealed that the two different types of crystals represent different morphologies of the same crystal form. The triangular prism-shaped crystals grew in the drop with the triangular face touching the cover slip, while the rods grew in the drop parallel to the cover slip, the apparent darker orange colour being an artefact caused by the orientation of the crystal in the drop under a microscope. The optimum crystallization conditions were achieved using a protein concentration of 20 mg ml^{-1} with solutions of 2.4 *M* ammonium sulfate and 0.1 *M* sodium acetate, pH 4.6, using 3 µl drops. The crystals usually appear within a few days to a week and continue growing for about a month, after which they are stable and can be kept for many months. Most crystals are of the rod-shaped form with the triangular prisms being seen only occasionally. Typical crystal sizes are $0.4 \times 0.1 \times 0.1$ mm although a few crystals have been grown up to 1.0 mm in length. Fig. 2 shows one of the largest crystals so far obtained.

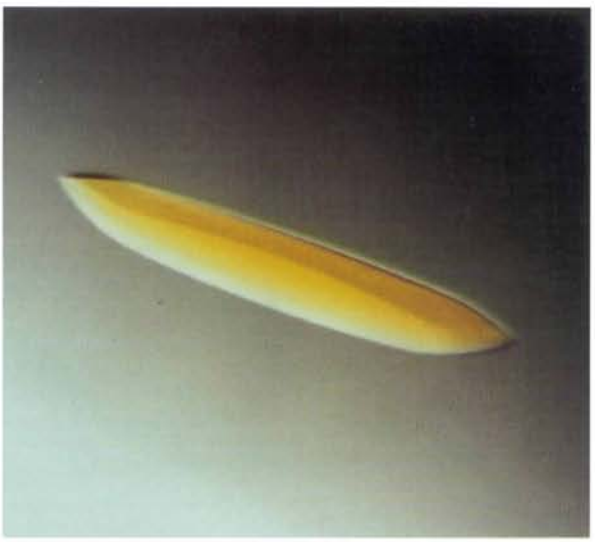

Fig. 2. A crystal of the *L. lactis* dihydroorotate dehydrogenase B heterotetramer. The crystal size is approximately $1.0 \times 0.15 \times 0.15$ mm.

To confirm that the crystals do contain both the PyrDB and the PyrK subunits, a large single crystal was washed in the crystallization buffer and dissolved in 25 ml of standard sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Two bands appeared from the crystal sample which corresponded to the two bands in the purified protein solution (Fig. 3). The estimated molecular weights of the PyrDB and the PyrK subunits are 34.5 and 31.5 kDa, respectively, in good agreement with their calculated molecular weights (33 and 29 kDa, respectively).

2.3. *X-ray diffraction analysis*

X-ray diffraction data collections have been carried out both in-house, using a conventional rotating-anode X-ray source, and with synchrotron radiation. Using the conventional source the diffraction limit for the largest and best crystals so far obtained was about 2.5 Å, although this was rare. Higher resolution data to about 2.0 Å resolution was attainable with synchrotron radiation.

A native data set was collected in-house to 2.6 Å resolution from a single large crystal of approximate size $1.0 \times 0.15 \times 0.15$ mm. This data collection was carried out at 288 K with an R-AXIS II imaging-plate system. X-rays were generated using a Rigaku RU-200 rotating copper anode operating at 50 kV and 180 mA. Monochromatic Cu $K\alpha$ X-rays ($\lambda = 1.54$ Å) were selected by a graphite monochromator and a 0.5 mm collimator. 58 diffraction images were recorded, each with an oscillation range of 1° and an exposure time of 45 min, during which time only slight radiation damage was seen. A total of 68 884 measurements of 19 174 unique reflections were recorded. The merged native data set has an $R_{\rm merge}$ of 9.6% and is 98.0% complete for reflections from 40 to 2.6 Å [79.3% complete for reflections where $I/\sigma(I) > 3$].

A 2.2 Å synchrotron native data set was collected from a single crystal of about $0.7 \times 0.2 \times 0.15$ mm. This data set was collected at station X11 at the EMBL Hamburg outstation with the crystal cooled to 277 K, using a 30 cm MAR Research image-plate detector and a beam wavelength of 0.912 Å. Exposures were measured by dose (typically 240 s) and only the central 18 cm of the plate was used. The crystal suffered badly from radiation damage and only 40 images with an oscillation range of 1° were suitable for processing. The merged data set comprises 75 830 measurements of 29 987 unique reflections, with an $R_{\rm merge}$ of 9.8%. This data set was 92.8% complete for reflections from 40 to 2.2 Å [56.2% complete for reflections where $I/\sigma(I) > 3$]. All diffraction images were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski, 1993), and structure factors were derived from the reflection intensities using the program *TRUNCATE* (Collaborative Computational Project, Number 4, 1994).

Analysis of the diffraction data revealed rhombohedral symmetry with the corresponding Laue group $\bar{3}m$. This uniquely determines the space group to be $R32$, with hexagonal unit-cell dimensions $a = b = 202.3$ and $c = 81.0$ Å. For an asymmetric unit containing one PyrDB subunit ($\simeq 33$ kDa) and one PyrK subunit ($\simeq 29$ kDa), the Matthews coefficient V_m (Matthews, 1968) is 2.57 Å^3 Da^{-1}, which gives an estimated crystal solvent content of 52%. Since the complex is known to be a functional tetramer, we would therefore expect it to be found on a crystallographic twofold axis.

We are currently in the process of screening heavy-atom derivative compounds in-house using soaking and co-crystallization experiments, with the aim of obtaining protein phases by multiple isomorphous replacement. The native synchrotron data set will enable us to refine the structure to high resolution.

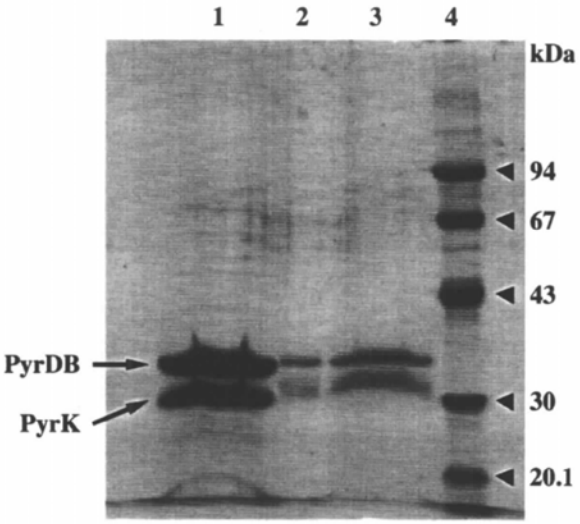

Fig. 3. SDS–PAGE analysis of a crystal of the heterotetrameric dihydroorotate dehydrogenase B. Lane 1 is the purified protein complex solution used for crystallization. Lanes 2 and 3 are from a dissolved crystal (5 and 20 µl, respectively). Lane 4 contains molecular weight standards. The SDS–PAGE system used was a modified version of that described by Fling & Gregerson (1986).

We would like to thank Mr Jens Christian Poulsen for valuable discussions and Mr Flemming Hansen for his help during data collection. We are grateful to the EMBL Hamburg outstation c/o DESY for beamtime on station X11, and thank Dr Wojcieck Rypniewski for his help during these experiments. The support from the Danish National Research Foundation is gratefully acknowledged and we thank the European Union for support of the work at EMBL Hamburg through the Human Capital and Mobility Programme Access to Large Installations project, contract number CHGE-CT93-0040.

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