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Purification, crystallization and X-ray diffraction analysis of dihydropyrimidinase from *Dictyostelium discoideum*

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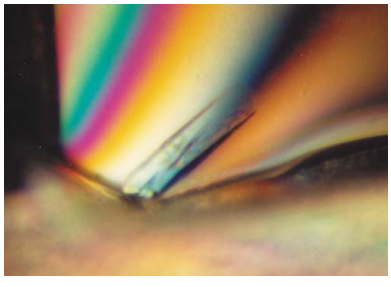

Dihydropyrimidinase (EC 3.5.2.2) is the second enzyme in the reductive pyrimidine-degradation pathway and catalyses the hydrolysis of 5,6-dihydrouracil and 5,6-dihydrothymine to the corresponding N-carbamylated β-amino acids. The recombinant enzyme from the slime mould *Dictyostelium discoideum* was overexpressed, purified and crystallized by the vapour-diffusion method. One crystal diffracted to better than 1.8 Å resolution on a synchrotron source and was shown to belong to space group $I222$, with unit-cell parameters $a = 84.6$, $b = 89.6$, $c = 134.9$ Å and one molecule in the asymmetric unit.

1. Introduction

Nucleotides play a central role in cellular energy metabolism and in a large number of biochemical processes and are the building blocks of the polynucleotides DNA and RNA. In mammalian tissue, the nucleotide bases uracil and thymine are degraded in the reductive pyrimidine-catabolic pathway, which comprises three enzymes: dihydropyrimidine dehydrogenase, dihydropyrimidinase (dihydropyrimidine amidohydrolase, DHPase; EC 3.5.2.2) and β-alanine synthase. Here, this pathway is the only metabolic route for the biosynthesis of β-alanine, which is a putative neurotransmitter owing to its chemical similarity to γ-aminobutyrate (Sandberg & Jacobson, 1981). DHPase catalyses the second step in the degradation of uracil and thymine, the reversible hydrolysis of 5,6-dihydrouracil and 5,6-dihydrothymine to the corresponding N-carbamylated β-amino acids *N*-carbamyl-β-alanine and *N*-carbamyl-β-aminoisobutyrate.

Enzymes of the reductive pyrimidine-degradation pathway are the major cause for the inactivation of clinically applied pyrimidines such as 5-fluorouracil (5-FU), which is used in the treatment of several tumours (Wasternack, 1980). Consequently, high doses of the drug have to be administered (Milano & Etienne, 1994), causing the accumulation of fluorinated products with severe side effects, especially to the nervous system (Okeda *et al.*, 1990). Additionally, together with the *de novo* biosynthetic and salvage pathways for pyrimidines, the catabolic pathway determines the amount of available pyrimidine nucleotides in the cell for nucleic acid synthesis, especially during cell proliferation (Ferdinandus *et al.*, 1971). Several genetic disorders of pyrimidine catabolism have been described with varying clinical phenotypes and are usually characterized by neurological disorders and a high risk of 5-FU toxicity (van Gennip *et al.*, 1997).

In mammals, DHPase is expressed in both liver and kidney (Traut & Loechel, 1984). The mammalian enzyme has been purified from different sources, such as bovine liver (Brooks *et al.*, 1983), calf liver (Kautz & Schnackerz, 1989), pig liver (Jahnke *et al.*, 1993) and rat liver (Traut & Loechel, 1984; Kikugawa *et al.*, 1994). Additionally, genes encoding DHPase have been cloned from human (Hamajima *et al.*, 1996) and rat liver (Matsuda *et al.*, 1996), *Caenorhabditis elegans* (Li *et al.*, 1992), *Arabidopsis thaliana*, *Drosophila melanogaster*, *Saccharomyces kluyveri* and *Dictyostelium discoideum* (Gojkovic *et al.*, 2000, 2003). Some of these have been expressed and partially purified as recombinant proteins. The purification and crystallization of DHPase from *S. kluyveri* has been described previously (Dobritzsch *et al.*, 2005).

All previously characterized DHPases are homotetrameric Zn^{2+}-containing metalloenzymes with two bound metal ions per subunit (Jahnke *et al.*, 1993; Gojkovic *et al.*, 2003; Kikugawa *et al.*, 1994; Kautz & Schnackerz, 1989; Brooks *et al.*, 1983). Molecular weights for the subunits range from 56 to 65 kDa. DHPases show sequence similarity to hydantoinases and have been shown to have a common evolutionary origin (Gojkovic *et al.*, 2003). However, the bacterial hydantoinases are apparently not involved in pyrimidine catabolism since most of them do not hydrolyze dihydropyrimidines (Kim & Kim, 1998; Syldatk *et al.*, 1999). Furthermore, DHPases possess sequence similarities to proteins involved in the development of the nervous system, such as DHP-related proteins (DRPs; Hamajima *et al.*, 1996) and collapsin-response-regulator protein (CRMP; Goshima *et al.*, 1995).

D. discoideum has previously been characterized for the presence of the reductive pyrimidine-degradation pathway (Gojkovic *et al.*, 2001, 2003). The primary sequence of DHPase from *D. discoideum* is 56% identical and 70% similar to that of the human enzyme. Since slime mould is more closely related to mammals than yeast, DHPase from *D. discoideum* represents a biochemically characterized enzyme which is so far the most similar to the human counterpart. In the absence of an available structure of human DHPase, the structure of the *D. discoideum* enzyme can serve as a model. Here, we report the purification, crystallization and X-ray diffraction analysis of DHPase from the slime mould *D. discoideum*.

2. Material and methods

2.1. Protein purification

DHPase from *D. discoideum* was cloned and expressed as a His_8-tagged fusion protein in *Escherichia coli* BL21 strain as described previously (Gojkovic *et al.*, 2003). The introduction of the tag (PGDDDDKHHHHHHHHSGD) extended the polypeptide chain on the C-terminus by 18 residues. This results in an overall protein of 521 amino acids with a molecular weight of 58.2 kDa per monomer. Harvested cells were resuspended in buffer *A* [50 m*M* sodium phosphate pH 7.0, 300 m*M* NaCl, 10%(*v*/*v*) glycerol, 1 m*M* DTT, 0.1 m*M* PMSF and one EDTA-free Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics) per 30 ml of buffer] and lysed by four passes in a French press at 6.9 MPa. Cell debris was removed by centrifugation at 24 500*g* for 30 min. To remove DNA, streptomycin was added to the supernatant to a final concentration of 2%(*w*/*v*) and stirred on ice for 30 min. The suspension was centrifuged at 24 500*g* for 30 min and the pellet discarded. For the removal of streptomycin, the supernatant was applied onto a gel-filtration G-25 column equilibrated with buffer *B* (as buffer *A* but without DTT and protease-inhibitor cocktail). Protein was eluted with buffer *B* at a flow rate of 2 ml min^{-1}. The total protein-containing fractions were pooled and subject to metal-affinity chromatography by applying the sample onto a 4 ml Ni–NTA column (Chelating Sepharose Fast Flow, Amersham Biosciences) equilibrated with buffer *B* containing 50 m*M* imidazole with the pH adjusted to 7.0. After washing the column with buffer *B* containing 50 m*M* imidazole, protein was eluted in a linear gradient of buffer *B* with 50–250 m*M* imidazole. Fractions containing DHPase were pooled and precipitated with ammonium sulfate (70% saturation at 273 K) to remove imidazole. The suspension was centrifuged at 24 500*g* for 30 min at 277 K and the supernatant was discarded. The pellet was dissolved in buffer *C* [100 m*M* sodium phosphate pH 7.0, 10%(*v*/*v*) glycerol] and applied onto a gel-filtration S-12 column equilibrated with buffer *C*. Elution of the protein was achieved at a flow rate of 0.5 ml min^{-1} with buffer *C*. Fractions containing *D. discoideum* DHPase were pooled, aliquoted and flash-frozen in liquid nitrogen for storage at 193 K.

For crystallization, protein was dialysed extensively against buffer containing 20 m*M* Tris–HCl pH 7.5, 1 m*M* DTT and 10%(*v*/*v*) glycerol. *D. discoideum* DHPase was concentrated by centrifugation at 6000*g* in a Microsep Centrifugal Concentrator (Pall Corporation) to a final concentration of 5.0 mg ml^{-1}. Finally, the protein sample was filtered by centrifugation in a Centrifugal Filter Unit (Amicon) with a 0.22 µm pore size.

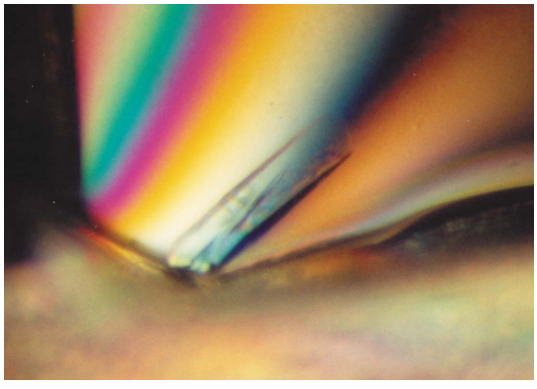

Figure 1
Crystal of *D. discoideum* DHPase (crystal form 1). The largest dimension of the crystal is about 0.5 mm.

2.2. Crystallization

Crystallization of *D. discoideum* DHPase was attempted with several commercially available sparse-matrix crystallization screens (Hampton Research, Molecular Dimensions, Emerald Biostructures) as well as systematic grid screens. Crystallizations were set up using the vapour-diffusion method with sitting drops in 96-well plates using 100 µl reservoir volume at 293 K. The drops contained 1 µl protein solution at 5 mg ml^{-1} mixed with 1 µl well solution. Protein crystals appeared under one screening condition, 0.1 *M* MMT buffer pH 7, 25%(*w*/*v*) PEG 1500 (crystal form 1), and one condition from a grid screen, 0.1 *M* MES pH 6.0, 1.0 *M* LiCl, 20%(*w*/*v*) PEG 6000 (crystal form 2). Only one *D. discoideum* DHPase crystal of form 1 appeared after 1 d, with dimensions of about 0.5 × 0.1 × 0.1 mm (Fig. 1). Crystal form 2 is considerably smaller, with maximum dimensions of about 0.10 × 0.02 × 0.02 mm, and appeared after about 30 d. Despite extensive efforts to reproduce crystals of form 1, this could not be achieved.

2.3. Data collection and processing

Diffraction data from both crystal forms of *D. discoideum* DHPase were collected at beamline ID14-4 at the ESRF (Grenoble, France) using an ADSC Quantum 4 detector. Data collection was accomplished on crystals frozen in a cold nitrogen stream at 100 K. Prior to freezing, the crystals were briefly soaked in a cryosolution containing mother liquor and 50%(*w*/*v*) PEG 1000 in 4:1 or 1:1 ratios for crystal form 1 and 2, respectively. Crystals of form 2 diffracted poorly to about 5 Å with diffuse scattering around the maxima and were discarded. The crystal of form 1 diffracted to better than 1.8 Å. However, the diffraction was very anisotropic, and additional diffuse scattering and a high mosaic spread of about 2° were observed. Diffraction data were collected at a crystal-to-detector distance of 175 mm using a wavelength of 0.939 Å. An oscillation angle of 0.25° was used to collect a total of 360° of data. Integration of the diffraction data was carried out with *MOSFLM* (Leslie, 1992).

Table 1
Diffraction and data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.16–2.05 Å).

Wavelength (Å)	0.939
Space group	*I*222
Unit-cell parameters (Å)	a = 84.6, b = 89.6, c = 134.9
Resolution (Å)	50–2.05
Total No. of reflections	94478 (12225)
Unique reflections	30568 (4408)
Multiplicity	3.1 (2.8)
Completeness (%)	94.4 (94.3)
Mean $I/\sigma(I)$	7.8 (2.1)
$R_{\rm sym}$(%)	13.4 (66.2)

Table 2
Sequence similarities to *D. discoideum* DHPase and molecular-replacement (MR) statistics (for space group *I*222 unless otherwise stated).

Protein	PDB code	Sequence identity (%)	Sequence similarity (%)	MR *R* factor (%)	MR CC (%)
CRMP/DRP-1	1kcx	51	69	43.2	55.0
D-Hydantoinase	1k1d	41	59	48.3	44.9
D-Hydantoinase	1gkq	39	57	49.3	39.9
D-Hydantoinase	1nfg	36	55	50.7	38.6
L-Hydantoinase	1gkr	26	47	55.7	24.0
CRMP/DRP-1†	1kcx	51	69	55.5	24.5

† Space group $I2_12_12_1$.

Intensities were merged and scaled using *SCALA* (Evans, 1997) and structure factors were calculated with *TRUNCATE* (French & Wilson, 1978).

3. Results

Autoindexing indicated that the crystal belongs to an orthorhombic class and the space group was determined to be *I*222 (or $I2_12_12_1$), with unit-cell parameters a = 84.6, b = 89.6, c = 134.9 Å. The Matthews coefficient (Matthews, 1968) was calculated to be 2.5 Å^3 Da^{-1} with a solvent content of 50%, assuming the presence of one molecule in the asymmetric unit. Since the quaternary structure of DHPase has been shown to be tetrameric (Jahnke *et al.*, 1993; Gojkovic *et al.*, 2003; Kikugawa *et al.*, 1994; Kautz & Schnackerz, 1989; Brooks *et al.*, 1983), it is likely that the biological oligomer is formed by the crystallographic 222 symmetry.

During data processing it became evident that the mosaicity is about 2.5° and it had to be fixed together with the box size to allow integration of the highly diffuse reflections. Furthermore, radiation damage became evident, so that only 125° of data could be used in the scaling process. Together with the anisotropy of the data, this made it necessary to cut the high-resolution limit of the data at 2.05 Å. These features could explain the relatively high $R_{\rm sym}$ of 13.4% observed for the whole data set. The statistics of the data collection are shown in Table 1.

Initial phases for *D. discoideum* DHPase were obtained by molecular replacement using the program *MOLREP* (Vagin & Teplyakov, 1997). Various structures of hydantoinases as well as a structure of a CRMP were deployed as search models. In the search, non-conserved amino acids were substituted by alanine. Molecular replacement was performed in both space groups, *I*222 and $I2_12_12_1$. For all search models a clear solution could be identified in space group *I*222, which generally displayed better statistics than corresponding solutions in space group $I2_12_12_1$. The results, together with the sequence identity, are shown in Table 2. These preliminary results indicate that *D. discoideum* DHPase is structurally more closely related to eukaryotic CRMP/DRPs than to prokaryotic hydantoinases. This confirms the previous results obtained by sequence-based phylogenetic analysis (Gojkovic *et al.*, 2003). Initial electron-density maps for the solution obtained with CRMP as a search model confirmed its correctness and will serve as a starting point for model building.

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