Crystallization of hamster dihydroorotase: involvement of a disulfide-linked tetrameric form

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Dihydroorotase (DHOase) catalyses the formation of L-dihydro-orotate (DHO) in the *de novo* pyrimidine biosynthetic pathway. The type I DHOase domain from hamster forms part of the trifunctional enzyme CAD. The hamster DHOase domain has been cloned and expressed in *Escherichia coli*. Solutions of the homodimeric protein convert to a homotetrameric species when incubated at ambient temperature. Formation of the tetrameric species is mediated *via* disulfide linkages between single free cysteine residues on the surface of each monomer. This process is also observed under conditions used for crystallization of the hamster DHOase domain; crystals composed exclusively of the tetrameric species grow from solutions containing as little as 10% tetramer. The crystallization of pure tetrameric DHOase results in two crystal forms: form I, with space group $C222_1$ and unit-cell parameters $a = 127.1$, $b = 603.5$, $c = 144.7$ Å, and form II, with space group $P2_1$ and unit-cell parameters $a = 260.5$, $b = 148.2$, $c = 308.0$ Å, $\beta = 102.2°$. Data have been recorded to 4.3 and 4.0 Å resolution, respectively.

1. Introduction

Dihydroorotase (DHOase; EC 3.5.2.3) is a zinc metalloenzyme that catalyses the reversible cyclization of *N*-carbamyl-L-aspartate (CA-asp) to DHO in the third step of the *de novo* pyrimidine biosynthetic pathway. In prokaryotes, DHOase is a homodimeric and monofunctional enzyme of approximate molecular weight 40 kDa (Washabaugh & Collins, 1984). In higher eukaryotes, DHOase activity is associated with a trifunctional enzyme which catalyses the first three steps of the pathway (Coleman *et al.*, 1977). CAD contains dihydroorotase, carbamyl phosphate synthetase (CPSase) and aspartate transcarbamylase (ATCase) activities arranged NH_3^+–CPSase–DHOase–bridge–ATCase–COO^- (Simmer *et al.*, 1990; Williams *et al.*, 1990).

Through extensive amino-acid sequence alignments, Holm & Sander (1997) proposed that DHOase belongs to the amidohydrolase superfamily of enzymes that catalyse a diverse set of hydrolytic reactions. The superfamily is divided into two subsets of enzymes predicted to have a common structural core consisting of eight alternating β-sheets/α-helices and a signature pattern of four conserved histidines and one aspartate residue. The first subset includes the enzymes urease and phosphotriesterase, which have binuclear metal centres in which the two metal ions are bridged by a solvent hydroxide and a carboxylated lysine residue. The second subset includes adenosine deaminase, which lacks the carboxylated lysine

residue and has a single five-coordinate zinc ion at the active site ligated by three conserved histidines, one aspartate residue and a water molecule (Wilson *et al.*, 1991). DHOase was originally proposed to belong to the second subset of the amidohydrolase superfamily because it was believed to bind a single divalent cation (Holm & Sander, 1997).

A recently reported phylogenetic analysis (Fields *et al.*, 1999) classifies the DHOases into two major classes thought to arise from ancestral gene duplication. Type I DHOases are the most ancient, are larger in size, are found in all domains of life (Eukarya, Eubacteria and Archaea) and include trifunctional (*e.g.* mammalian CAD) and monofunctional enzymes (*e.g.* *Bacillus*, *Lactobacillus* and *Streptococcus*). Type II DHOases (*e.g.* that from *Escherichia coli*) are predominately eubacterial and exhibit significant differences in their primary amino-acid sequences. They are predicted to have secondary-structural characteristics similar to the those of type I DHOases. These enzymes are generally smaller, being 50 and ten residues shorter at the amino and carboxyl termini, respectively, with several internal insertions and deletions compared with the type I enzymes.

The first structure of a DHOase (that from *E. coli*) has been reported to a resolution of 1.7 Å and has a binuclear metal centre (Thoden *et al.*, 2001). In agreement with the predictions of Holm & Sander (1997), the overall architecture of the enzyme resembles that of urease. The protein folds into a TIM-

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barrel motif, with eight strands of parallel β-sheet flanked on the outer surface by α-helices. Interestingly, the homodimeric structure showed DHO bound at the active site of one subunit, while CA-asp was located in the active site of the other subunit.

Hamster DHOase is part of the trifunctional enzyme CAD and has been classified as a type I DHOase (Fields *et al.*, 1999). Biochemical studies indicate that this class has one catalytic Zn atom per subunit (Kelly *et al.*, 1986; Zimmermann *et al.*, 1995; Huang *et al.*, 1999). In contrast, the structure of *E. coli* DHOase (a type II enzyme) contains a binuclear metal centre with two Zn^{II} ions bridged by a carboxylated lysine residue and a solvent molecule (Thoden *et al.*, 2001). Although hamster and *E. coli* DHOases share only 15% sequence identity, closer inspection of the sequence of hamster DHOase shows that Lys101 could be carboxylated in a similar manner to Lys102 in *E. coli* DHOase. The metal-coordinating residues also appear to be conserved.

We wish to report here an interesting phenomenon relating to the crystallization of the hamster DHOase domain. Solutions of the homodimeric DHOase domain convert to a tetrameric species when incubated at ambient temperature. It is this tetrameric species which crystallizes, even from solutions containing as little as 10% tetramer. Pure solutions of the tetrameric DHOase domain crystallize in two crystal forms from which low-resolution data sets have been collected.

2. Experimental procedures

2.1. Protein expression and purification

The plasmid pCW25, encoding the hamster DHOase domain with an additional 33 amino-acid residues extending from the carboxyl terminus into the bridge, was used to transform into *E. coli* K strain SØ1263/pyrC$^-$. The recombinant *E. coli* was grown and the hamster DHOase overexpressed and purified as described previously (Williams *et al.*, 1990). The enzyme domain was further purified by loading onto a POROS-HQ column (Perceptive Biosystems, Foster City, USA), which was eluted with an NaCl gradient (0–1 *M*). Fractions containing hamster DHOase were pooled, concentrated, desalted and stored in enzyme buffer [20 m*M* sodium HEPES pH 7.3, 10%(*v*/*v*) glycerol, 0.1 m*M* EDTA and 1 m*M* DTT].

To examine the association state of the DHOase domain, protein solutions or washed dissolved crystals were analyzed by native PAGE using a Mighty Small II SE 250 vertical slab unit (Hoefer Scientific Instrument, San Francisco, USA) or PhastSystem with an 8–25% Phastgel gradient (Pharmacia Biotech, Uppsala, Sweden). Samples were mixed with an equal volume of loading buffer [62.5 m*M* Tris–HCl pH 6.8, 10%(*v*/*v*) glycerol, 0.1%(*w*/*v*) bromophenol blue] before analysis. Cross-linked bovine albumin (monomer, 66 kDa; dimer, 132 kDa; trimer, 198 kDa) was used as a standard.

The molecular weight of the tetrameric DHOase species was estimated by size-exclusion chromatography on a Superdex-200 column (Pharmacia Biotech, Uppsala, Sweden). A calibration curve was constructed using protein standards (blue dextran, 2000 kDa; ferritin, 440 kDa; aldolase, 158 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa).

Quantification of the number of free cysteine residues present on the surface of the dimeric and tetrameric DHOase species was determined by titration with 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB). The reaction was initiated by addition of DTNB (0.15 m*M*) to a DHOase sample (10 nmol, 50 m*M* sodium HEPES pH 7.4), with release of the 5-thio-2-nitrobenzoate dianion (TNB^{2-}) being monitored at 412 nm. The number of free cysteine residues was calculated using ε_{412nm} (TNB^{2-}) = 14 150 M cm^{-1}.

The free surface cysteine residue present in dimeric DHOase was identified by mass-spectroscopic analysis of cleavage products following cyanylation with 2-nitro-5-thiocyanobenzoic acid (NTCB). A DHOase sample (10 µ*M*) was added to a mixture containing NTCB [200 µ*M*; 0.1 *M* Tris–HCl pH 8.0, 10% (*v*/*v*) glycerol] and the reaction was monitored by following the release of TNB^{2-} at 412 nm. After reaction, the cyanylated DHOase was cleaved by increasing the pH of the mixture to pH 9.5 with NaOH (1.0 *M*). The mixture was incubated at 310 K for 2–3 d. The sample was then desalted by HPLC on a Jupiter 5µ C4 column (Phenomenex, CA, USA), which was eluted with an acetonitrile gradient [5–95% in 0.1%(*v*/*v*) acetic acid and 0.02%(*v*/*v*) TFA]. The eluate was analyzed by electrospray ionization mass spectrometry (LCQ, ThermoFinnigan, CA, USA).

To prepare the C241S mutant of hamster DHOase, the DHOase domain was amplified by PCR from the pCW25 plasmid using four primers: the forward and reverse primers for the hamster DHOase domain, which contained *Xba*I (5′-GCCGCTCTA-GAAATAATTTTGTTTAACTTTAAGAA-GGAGTATATCCATGACT-3′) and *Bam*HI cleavage sites (5′-GTCCGGATCCTTAT-GTCGTGGTTATTTCTGTGGTGGCAG-GAG-3′), and the forward and reverse primers for the C241S mutation, 5′-CTGG-AGGAGAAG**TCT**GGGCCCAAG-3′ and 5′-CTTGGGCCC**AGA**CTTCTCCTCCAG-3′, respectively. The resulting C241S PCR product was cloned into the pET3d expression vector to yield pCW25/C241S, which was then transformed into the *E. coli* BL21(DE3) strain. C241S hamster DHOase was overexpressed and purified using the same procedure as for the native recombinant hamster DHOase domain.

Large-scale preparation of the pure tetrameric DHOase domain was achieved by incubating pure dimeric DHOase [0.4–0.8 mg ml^{-1}; 20 m*M* sodium HEPES pH 7.3, 10%(*v*/*v*) glycerol, 0.1 m*M* EDTA 0.15 *M* NaCl and 0.1 m*M* DTT] for 3–4 weeks at 298 K. The tetrameric species was then purified by size-exclusion chromatography on a Superdex-200 column (Pharmacia Biotech, Uppsala, Sweden).

DHOase was assayed in the degradative direction as described previously (Christopherson *et al.*, 1989). Reaction mixtures (23 µl) containing 50 m*M* sodium HEPES pH 7.4 and 5% glycerol with varying concentrations of L-(2-^{14}C)DHO (2–200 µ*M*, 54 Ci mol^{-1}) were preincubated at 310 K for 5 min. Reactions were initiated by adding pure DHOase (2 µl, 10 ng protein), and aliquots (7 µl) were spotted onto poly(ethyleneimine)-cellulose chromatograms at 5 min intervals. The ^{14}C-labelled CA-asp produced was separated from DHO by ascending chromatography with ~0.34 *M* LiCl. The ^{14}C contents of the substrate and product spots were determined with a Typhoon 8600 (Molecular Dynamics, Sunnyvale, USA) and *Imagequant* software. Reaction velocities were determined by linear regression to these three time points and K_M and V_{max} values were calculated by nonlinear regression to the Michaelis–Menten equation using *Sigma-Plot* (Jandel Scientific, Corte Madera, CA, USA).

2.2. Crystal growth and analysis

Crystallization conditions were screened at 293 K according to the sparse-matrix method (Jancarik & Kim, 1991) using commercially available buffers (Hampton Research, Laguna Hills, California, USA) and the hanging-drop vapour-diffusion technique. Small crystals were observed under one condition only: in 4.0 *M* sodium formate. Parameters such as buffer compo-

sition, pH, temperature and protein concentration were subsequently varied to improve crystal quality. Flat oval-shaped crystals of 100 μm in the maximum dimension were grown by mixing 2.0 μl of protein solution [10 mg ml^{-1}; 20 mM sodium HEPES pH 7.3, 10%(v/v) glycerol, 0.1 mM EDTA, 1 mM DTT] with an equal volume of reservoir solution [15%(w/v) PEG 4K, 1.6 M sodium formate and 0.1 M sodium HEPES pH 7.5] and equilibrating this drop against 1.0 ml of reservoir solution at 293 K.

Crystallization trials continued with two different preparations of the tetrameric DHOase domain (batches 1 and 2) distinguished by the presence of a contaminating high-molecular-weight species (Fig. 1). Two different crystal forms of the DHOase domain were grown. Form I crystals (tetrameric species, batch 1) were grown using 14%(w/v) PEG 4K, 1.6 M sodium formate, 0.1 M sodium HEPES pH 7.6 and had typical dimensions of 100 × 100 × 100 μm; form II crystals (tetrameric species, batch 2) were grown using 10%(w/v) PEG 4K, 1.4 M sodium formate, 0.1 M sodium HEPES pH

Figure 1
Native PAGE analysis of DHOase. (*a*) Analysis of the crystallization drop: lane 1, protein from mother liquor; lane 2, dissolved crystals (composed exclusively of tetrameric DHOase). Migration distances for BSA standards are indicated. (*b*) Protein preparations for crystal forms I and II: lane 1, protein batch 1 (crystal form I); lane 2, protein batch 2 (crystal form II).

7.6 and had typical dimensions of 500 × 100 × 100 μm (Fig. 2).

Crystals were cryoprotected by successive soaking in reservoir solutions containing increasing quantities of glycerol [the final concentration of glycerol was 15%(v/v), achieved in 5% steps] and flash-frozen in liquid nitrogen for data collection. Data were collected as follows. For crystal form I, data to 4.3 Å resolution were recorded at 110 K on beamline 9-2 at the Stanford Synchrotron Radiation Laboratory; for crystal form II, data to 4.0 Å resolution were recorded at 110 K on BioCARS beamline 14-C at the Advanced Photon Source, Argonne National Laboratory. All data were integrated, scaled and merged using the *HKL* suite (Otwinowski & Minor, 1991).

3. Results and discussion

The hamster DHOase domain exists as a homodimer in solution (Kelly *et al.*, 1986). The recombinant hamster DHOase domain was shown to be a homodimer by analytical ultracentrifugation (Williams *et al.*, 1990). Metal-substitution experiments with hamster dihydroorotase require that the protein sample be incubated at 277 K for approximately two weeks (Huang *et al.*, 1999). Concentrated (∼30 mg ml^{-1}) samples from this procedure were analyzed by native PAGE. Surprisingly, in addition to the expected dimeric DHOase, a new species with an apparent molecular weight of 170 kDa was observed (Fig. 1). Using size-exclusion chromatography (data not shown) this species had a molecular weight of approximately 190 kDa, which is in the vicinity of the expected size of a putative tetrameric form of DHOase (165 kDa). Using the relative peak heights of the dimeric and tetrameric species, approximately 5% of the sample was converted to the tetrameric species after incubation for two weeks.

The analysis of dimeric and tetrameric DHOase samples by SDS–PAGE in the absence of reducing agents revealed single bands with apparent molecular weights of 40 and 75 kDa, respectively (data not shown), indicating that the tetrameric DHOase is linked *via* disulfide bond(s). The sequence of dimeric DHOase includes six cysteine residues. Titration of a sample of dimeric DHOase with DTNB gave an absorbance change at 412 nm corresponding to a single free surface cysteine residue per monomer. By contrast, analysis of tetrameric DHOase gave no free surface cysteines. Therefore, the tetrameric form of DHOase results from the formation of two disulfide linkages

between two dimeric DHOases *via* the single exposed cysteine residue located on each subunit.

Cleavage and subsequent mass-spectroscopic analysis of dimeric DHOase was used to identify the cysteine residue involved in the formation of tetrameric DHOase. Titration of dimeric DHOase with NTCB under native conditions confirmed the presence of one exposed cysteine residue per subunit. This cysteine residue was cyanylated and the polypeptide was cleaved under alkaline conditions at the amino peptide bond adjacent to the modified cysteine. The resultant two polypeptides were analyzed by mass spectrometry. One species had a molecular weight of 15 440 Da, consistent with cleavage of the protein at Cys241. Plasmid pCW25/C241S with Cys241 mutated to serine was transformed into *E. coli* BL21(DE3) and overexpressed and purified. This DHOase mutant showed an enzymic activity similar to that of the native DHOase, but had no detectable exposed
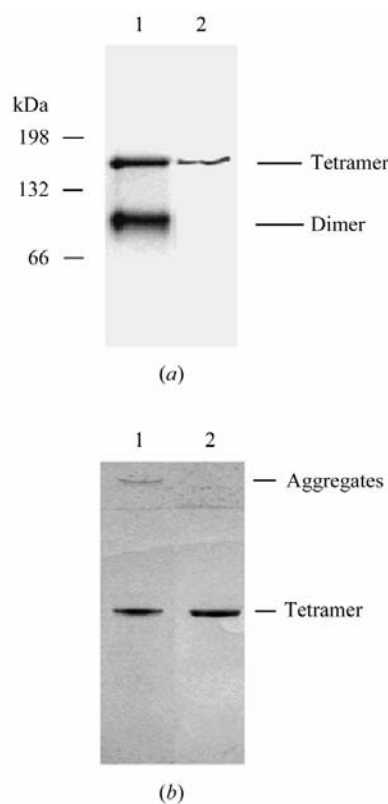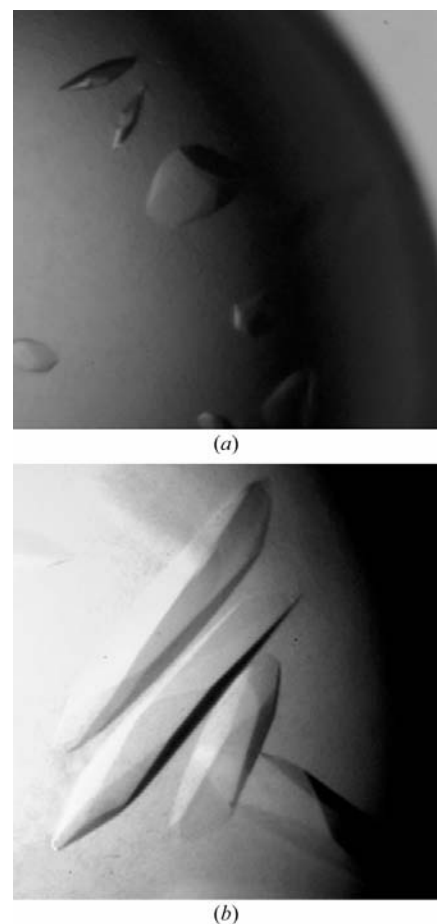

Figure 2
Crystals of DHOase. (*a*) Crystal form I. The largest crystal, at the centre of the drop, has dimensions of 100 × 100 × 100 μm. (*b*) Crystal form II. Typical crystal dimensions are 500 × 100 × 100 μm.

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Table 1
Data-collection statistics.

Values for the highest shell are given in parentheses.

	Form I	Form II
Space group	$C222_1$	$P2_1$
Unit-cell parameters		
a (Å)	127.1	260.5
b (Å)	603.5	148.2
c (Å)	144.7	308.0
β (°)		102.2
Resolution (Å)	4.3	4.0
Mosaicity (°)	0.67	0.35
Observations	724134	4520791
Unique reflections	39560	93055
Redundancy	3.0 (3.0)	1.9 (1.8)
Completeness (%)	96.7 (98.1)	95.8 (95.1)
$I/\sigma(I)$	8.9 (2.3)	9.7 (2.0)
R_{merge}	0.10 (0.41)	0.05 (0.26)

cysteine residues when titrated with DTNB under native conditions. C241S DHOase does not form the tetrameric species.

Initial attempts to crystallize the DHOase domain resulted in flat oval-shaped crystals which appeared over 2–3 months. These crystals were dissolved and analyzed by native PAGE, indicating exclusively the tetrameric DHOase species (Fig. 1). The protein preparation used to grow these crystals contained approximately 10% tetramer.

With a view to preparing samples of pure tetrameric DHOase for crystallization, optimal conditions for large-scale preparation of this species were investigated. We found that incubation of dimeric DHOase at a concentration of 0.4–0.8 mg ml^{-1} yielded approximately 60–70% conversion into the tetramer after 3–4 weeks at 298 K. The tetrameric species was then purified by size-exclusion chromatography. Tetrameric DHOase is less active than its dimeric form, with V_{max} of 1.93 ± 0.1 µmol min^{-1}mg^{-1} and K_{M} for DHO of 41 ± 5 µM, respectively, compared with a V_{max} of 3.9 ± 0.5 µmol min^{-1}mg^{-1} and a K_{M} of 22 ± 4.7 µM for dimeric DHOase.

Crystallization screens carried out with two different batches of tetrameric DHOase, distinguished by the presence of a contaminating high-molecular-weight species (Fig. 1), resulted in two different crystal forms which grew over 2–3 weeks (Fig. 2). Both forms grew under similar conditions with PEG 4K (10–15%), sodium formate (1.4–1.6 *M*) and sodium HEPES buffer pH 7.6 at 293 K. Complete native data sets from both forms were collected using synchrotron radiation. The data-collection statistics are summarized in Table 1. Form I crystals belonged to space group $C222_1$, with unit-cell parameters a = 127.1, b = 603.5, c = 144.7 Å. Form II crystals belonged to space group $P2_1$, with unit-cell parameters a = 260.5, b = 148.2, c = 308.0 Å, β = 102.2°. Using the Matthews formula (Matthews, 1968), it is possible that the asymmetric unit of crystal form I contains two or three tetrameric molecules of DHOase (71 or 57% solvent; V_{M} = 4.3 or 2.9 Å^3 Da^{-1}, respectively) and that the asymmetric unit of crystal form II contains 8–12 tetramers (72–58% solvent; V_{M} = 4.5–3.0 Å^3 Da^{-1}).

4. Conclusions

Incubation of the recombinant dimeric hamster DHOase domain at ambient temperature results in the slow formation of a disulfide-linked tetrameric species. This form of the protein is not found in cells, since the DHOase domain of the trifunctional enzyme CAD is dimeric in solution (Kelly *et al.*, 1986). In addition, the tetrameric species is less active than the dimeric form, with $V_{\mathrm{max}}/K_{\mathrm{M}}$ showing a fourfold decrease.

Formation of the tetramer has improved crystallization of the hamster DHOase domain. Crystals of the tetramer grew over a period of 2–3 months from a preparation containing approximately 10% of this form. Crystallization trials using pure tetrameric DHOase resulted in two crystal forms which grew over a period of weeks. Complete data sets at low resolution have been recorded from both crystal forms.

We aim to solve the structure of the hamster DHOase domain by the multiple-wavelength anomalous dispersion technique using the intrinsic Zn atoms. The unit-cell parameters of these crystals, particularly those of crystal form II, make these large crystallographic problems. Screening of crystallization conditions is continuing so that higher resolution data may be obtained. In addition, crystallization of the C241S mutant (which does not form the tetramer) has begun in order to solve the structure of the biologically relevant dimeric form of the DHOase domain.

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