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Crystallization and preliminary X-ray crystallographic analysis of the *Pseudomonas aeruginosa* cyclohexadienyl dehydratase

Palangpon Kongsaeree,[a] Jun Liang,[a] Roy A. Jensen[b] and Jon Clardy[a]*

[a]Department of Chemistry, Cornell University, Ithaca, New York 14853-1301, USA, and [b]Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611-0700, USA

Correspondence e-mail: jcc12@cornell.edu

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The title protein has been crystallized in a new crystal form. The crystals belong to the cubic space group $P4_132$ (or $P4_332$) with unit-cell dimensions $a = b = c = 126.1$ Å at 100 K and typically diffract beyond 1.6 Å at the Cornell High Energy Synchotron Source (CHESS) A1 beamline.

1. Introduction

In the biosynthesis of phenylalanine, chorismate is converted to phenylalanine by alternative pathways that differ by having either phenylpyruvate or arogenate (Herrmann & Somerville, 1983) as an intermediate (Fig. 1). Some organisms, such as *Pseudomonas aeruginosa* and *Xanthomonas campestris*, use both pathways, while other organisms, such as *Escherichia coli*, use only one (Patel *et al.*, 1977; Byng *et al.*, 1983; Whitaker *et al.*, 1984). In *E. coli*, chorismate from the shikimate pathway is converted to phenylpyruvate by the bifunctional P-protein, consisting of a chorismate mutase domain which converts chorismate to prephenate and a prephenate dehydratase domain which converts prephenate to phenylpyruvate (Calhoun *et al.*, 1973). Subsequently, phenylpyruvate is transaminated by phenylpyruvate aminotransferase to L-phenylalanine.

In *P. aeruginosa*, a monofunctional chorismate mutase (CM-F) produces prephenate, which has two alternative routes to phenylalanine: (i) transamination to L-arogenate by arogenate aminotransferase followed by conversion to phenylalanine by a monofunctional cyclohexadienyl dehydratase (CDT) or by arogenate dehydratase, or (ii) conversion to phenylpyruvate by CDT followed by conversion to phenylalanine by phenylpyruvate aminotransferase. Unlike the bifunctional P-protein, both enzymes in the arogenate pathway are insensitive to the feedback inhibition of phenylalanine, tyrosine or tryptophan, making it an overflow pathway for the phenylalanine synthesis (Fiske *et al.*, 1983; Zhao *et al.*, 1992).

Cyclohexadienyl dehydratase in *P. aeruginosa* (E.C. 4.2.1.51) exists as a homodimer (Zhao *et al.*, 1992) of a 28 kDa polypeptide chain (Zhao *et al.*, 1993). The gene encoding CDT, denoted *PheC*, was cloned from *P.*

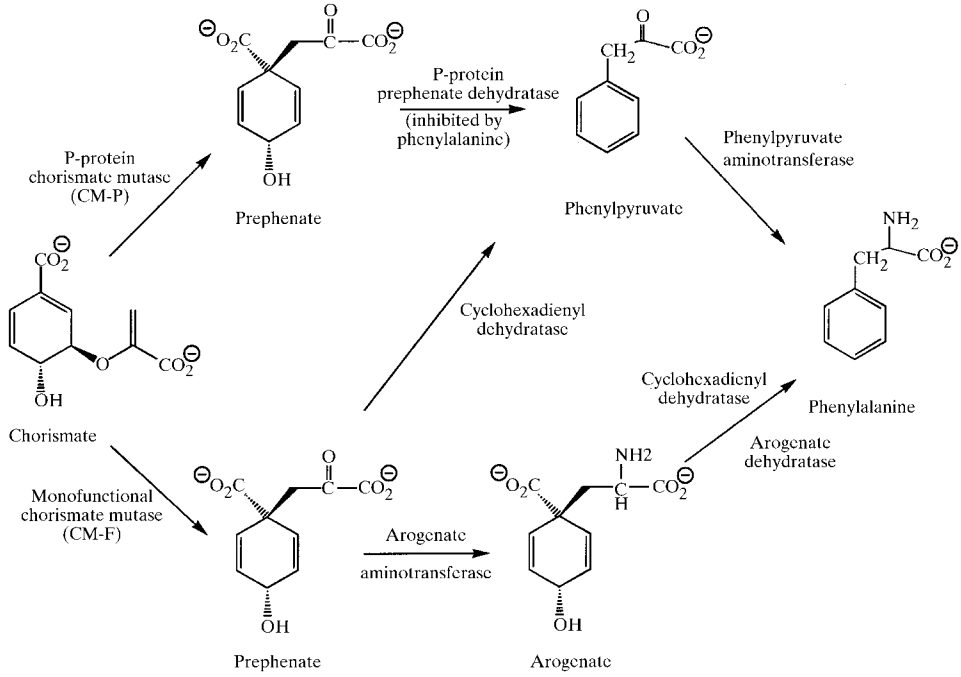

Figure 1
The dual pathways for L-phenylalanine biosynthesis.

aeruginosa and was expressed in *E. coli* with the use of high-copy number of pUC18. *P. aeruginosa* CDT, expressed in *E. coli* JP2255 carrying the pJZ1g plasmid, was purified to homogeneity (Obmolova *et al.*, 1993). As deduced from the 807 base-pair *PheC* gene, CDT consists of 268 amino acids (Fig. 2). CDT possesses both prephenate dehydratase and arogenate dehydratase activities as a result of broad substrate specificity for prephenate and arogenate. Prephenate is the better substrate for CDT with an activity ratio of 3.1:1 between prephenate dehydratase and arogenate dehydratase activity. The K_m value of CDT is 0.42 m*M* for prephenate and 0.22 m*M* for arogenate (Zhao *et al.*, 1992).

It has recently been shown that the *P. aeruginosa* CDT is a periplasmic protein (Zhao *et al.*, 1993). The first 25 N-terminal residues are a typical signal sequence for periplasmic proteins. The N-terminal sequencing of the purified protein yielded eleven consecutive residues that completely matched residues 26–36 as deduced from the *PheC* gene. Taking into account the removable 25 N-terminal residues, the functional CDT consists of 242 residues with a calculated molecular mass of 28 kDa. Therefore, it is possible that CDT is expressed as a proenzyme and released following spheroplast formation, osmotic shock or chloroform treatment. A role for CDT in the periplasm has not been identified. Note that CM-F and CDT are periplasmic proteins and P-proteins are cytosolic proteins.

2. Experimental

2.1. Cell cultures and protein purification

The protocols for cell cultures and protein purification were carried out with some optimization from the recently published protocol (Zhao *et al.*, 1992). *E. coli* JP2255 cells carrying the subclone pJZ1g were grown in 10 l LB media with 100 mg l^{-1} of ampicillin at 310 K. After 6–8 h, when the OD_{600} had reached 1.6–1.8, the cells were harvested by centrifugation at 3000 rev min^{-1} for 20 min. All subsequent steps were carried out either at 277 K or on ice. After the cell pellets were resuspended in 20 m*M* potassium phosphate buffer pH 8.2, 0.5 m*M* EDTA, 0.5 m*M* DTT (buffer *A*), PMSF was added to a final concentration of 1 m*M* and the suspended cells were disrupted by sonication. The suspension of the disrupted cells was centrifuged at 12000 rev min^{-1} for 20 min and the supernatant was then loaded onto an anion-exchange Q-Sepharose column, which had previously been equilibrated with buffer *A*. The anion-exchange column was washed with 2–3 column volumes of the equilibrating solution and eluted with a linear gradient of 0–300 m*M* potassium chloride at pH 8.2 in 2 × 350 ml. The active fractions, checked by absorbance at 280 nm and by prephenate dehydratase activity assays, were pooled. The active fractions were then concentrated several fold and washed with 20 m*M* potassium phosphate pH 7.2, 0.5 m*M* DTT, 0.5 m*M* EDTA solution (buffer *B*) twice. The concentrated sample was then loaded onto a hydroxylapatite (HAP) column (from Biorad) which had been previously equilibrated with buffer *B*. After washing with two column volumes of buffer *B*, the column was eluted with a 700 ml linear gradient of 20–300 m*M* potassium phosphate buffer pH 7.2. Again, fractions were collected and those with high cyclohexadienyl dehydratase activity were pooled together.

In the next step, instead of using Sephadex G-200 chromatography, a high-resolution anion-exchange Poros 10HQ (or 20HQ) column on the Biocad-Sprint PerSeptive HPLC system was used in order to speed up the purification. The active fractions from the hydroxylapatite column were concentrated and desalted by dialysis against a solution of 10 m*M* potassium phosphate pH 8.0, 10% glycerol, 0.5 m*M* DTT and 0.5 m*M* EDTA. On the HQ column, a linear gradient of 50–250 m*M* NaCl in 20 column volumes of 10 m*M* potassium phosphate buffer pH 8.0 with a flow-rate of 5 ml min^{-1} was used in the elution step, in which a single peak exhibiting high cyclohexadienyl dehydratase activity was obtained. The active fractions gave one single band on Coomassie-blue stained SDS–PAGE. The purified protein sample was dialyzed against a 20 m*M* potassium phosphate pH 8.0, 0.5 m*M* EDTA, 0.5 m*M* DTT, 10%(*v*/*v*) glycerol, 100 m*M* NaCl solution and subsequently concentrated to 10 mg ml^{-1} and checked by the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) as a standard. A total yield of over 100 mg of pure CDT was obtained from a 10 l culture.

2.2. Crystallization

CDT was originally crystallized (Obmolova *et al.*, 1993) in the tetragonal space group $P4_12_12$ or $P4_32_12$ using a high ammonium sulfate concentration at low pH. These crystals diffracted to 3 Å resolution. We were able to obtain better quality crystals using the low molecular weight polymers polypropylene glycol 400 (PPG 400) or polyethylene glycol 400 (PEG 400).

Small single crystals of CDT, with a perfect cubic morphology, were discovered using PPG 400 and pH-screening grids. Crystals appeared in 3 d using hanging drops containing 42% PPG 400, 100 m*M* Tris pH 8.2 at 277 K. These crystals typically grew to 0.1 × 0.1 × 0.1 mm. Replacement of PPG 400 with PEG 400 gave larger crystals. Cubic crystals of CDT, grown under the conditions 42% PEG 400 and 100 m*M* Tris pH 8.3, can reach 0.4 mm in each dimension.

The CDT crystals are stable in the crystallization drop at 277 K for at least a few months. However, the crystals are quite sensitive to temperature changes. If a crystallization tray is removed from the 277 K incubator and left at room temperature, a precipitate deposits on the crystals. Changes in temperature cause large crystals to quickly develop cracks. Although small crystals do not visibly crack, they noticeably lose diffraction power. The sensitivity of CDT crystals to temperature change can be alleviated by transferring the crystals to an artificial mother liquor containing 50% PEG 400, 100 m*M* Tris pH 8.2 and 100 m*M* NaCl. Crystals can be stored in this way at 277 K for at least a few months. The crystals are also thus protected from temperature change. It should be noted that glycerol plays a role in the crystallization of CDT, and under the PEG 400 conditions the protein will not crystallize in the absence of glycerol.

MPKSFRHLVQ ALACLALLAS ASLQAQESRL DRILESGVLR VTTTGDYKPF
SYRTEEGGYA GFDVDMAQRL AESLGAKLVV VPTSWPNLMR DFADDRFDIA
MSGISINLER QRQAHFSIPY LRNSKTPITL CSEEARFQTL EQIDQPGVTA
IVNPGGTNEK FARANLKKAR ILVHPDNVTI FQQIVDGKAD LMMTDAIEAR
LQSRLHPELC AVHPQQPFDF AEKAYLLPRD EAFKRYVDQW LHIAEQSGLL
RQRMEHWLEY RWPTAHGK

Figure 2
Amino-acid sequence of cyclohexadienyl dehydratase in *P. aeruginosa* deduced from the *PheC* gene. The first 25 amino acids (highlighted) are a typical signal sequence for periplasmic proteins.

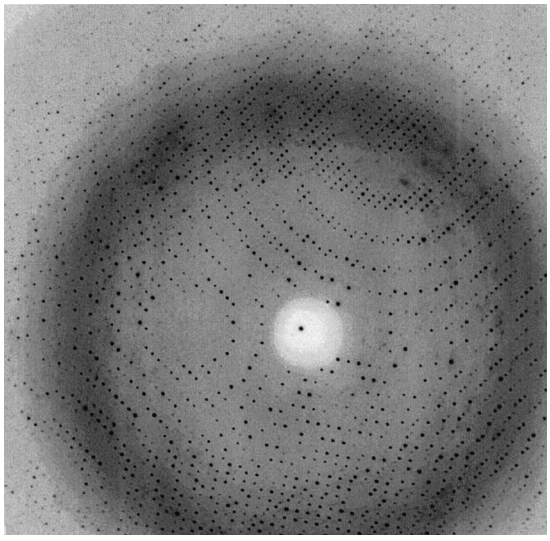

Figure 3
A 0.8° oscillation image of a native CDT crystal taken at the CHESS A1 beamline using a 1k CCD detector (ADSC). The crystal-to-detector distance was 120 mm and the wavelength of the X-ray beam was 0.91 Å. The resolution at the edge of the detector was around 2.1 Å.

2.3. Data collection and processing

A well frozen CDT crystal (0.3 × 0.3 × 0.3 mm) diffracted beyond 1.6 Å resolution on the A1 beamline at the Cornell High Energy Synchrotron Source (CHESS) using a wavelength of 0.91 Å and an ADSC 1k CCD detector. Crystals were mounted directly from the stabilizing solution with a nylon loop and flash-frozen in the cryogenic nitrogen stream. In order to balance spot separation and resolution on the 1k × 1k detector, data to only 1.8 Å resolution were collected. Data collection was performed using 0.8° oscillations and 15 s exposure time per frame, and a total of 94 frames (75.2°) were taken. Diffraction images were processed using *DENZO* and *SCALEPACK* (Minor, 1993; Otwinowski, 1993). CDT crystals from PEG 400 belong to the primitive cubic space group $P4_132$ or $P4_332$ with cell dimensions $a = b = c = 126.1$ Å. Assuming one CDT molecule per asymmetric unit, the calculated solvent content is 58% ($V_m = 2.9$ Å^3 Da^{-1}). The data set was of good quality with an R_{sym} of 5.2% (23.0% at 1.8 Å), a completeness of 92.8% (65% at 1.8 Å) and 29959 unique reflections from a total of 122720 measured reflections. This new crystal form has very low mosaicity, and the refined mosaicity from *SCALEPACK* is 0.2° for this data set.

More than 50 heavy-atom derivative data sets were taken and attempts to determine the heavy-atom sites are under way. In addition, SeMet-CDT has also been purified and crystallized. The SeMet-CDT crystal diffracts beyond 2.5 Å resolution at the CHESS A1 beamline. The structure determination will be reported in the future.

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