Crystallization and preliminary X-ray Crystallographic analysis of carbamoyl phosphate synthetase

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Abstract

Carbamoyl phosphate synthetase catalyzes the formation of carbamoyl phosphate, a high-energy intermediate used in several biosynthetic pathways. The enzyme from *Escherichia coli* has been crystallized at pH 8 in the presence of L-ornithine, $MnCl_2$ and ADP, using PEG 8000 in combination with NEt_4Cl and KCl. The crystals (apparently) belong to the orthorhombic space group $P2_12_12_1$ with unit-cell dimensions of $a = 154.4$, $b = 166.5$ and $c = 338.7$ Å. The crystals are relatively sensitive to radiation damage, but show diffraction to beyond 2.8 Å resolution. A low-resolution (3.5 Å) native data set has been recorded and conditions for flash cooling the crystal have been established.

Introduction

Carbamoyl phosphate synthetase (CPS) from *Escherichia coli* catalyzes the formation of carbamoyl phosphate from glutamine, bicarbonate, and ATP as shown below, and is one of the first steps in the biosynthesis of pyrimidines.

$$Gln + bicarbonate + 2MgATP$$
$$\rightarrow Glu + 2MgADP + P_i + carbamoyl\ phosphate$$
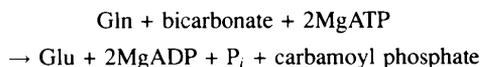
Ammonia can also be utilized as a nitrogen source by this enzyme. The synthesis of carbamoyl phosphate involves the activation of bicarbonate through the formation of carboxyphosphate, which then undergoes nucleophilic attack by ammonia to form carbamate. The carbamate intermediate is then phosphorylated by the second molecule of ATP to give the final product carbamoyl phosphate (Meister, 1989; Raushel, Anderson & Villafranca, 1978). Carbamoyl phosphate is subsequently used in two biosynthetic pathways. It may react with aspartate, in a reaction catalyzed by aspartate transcarbamoylase, for the eventual synthesis of pyrimidine nucleotides, or it may react with ornithine for the subsequent synthesis of arginine and/or urea. The working scheme for the synthesis of carbamoyl phosphate is shown below (Anderson & Meister, 1965).

(I)
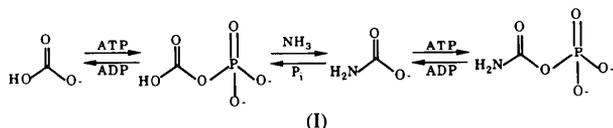
Evidence for this has been experimentally obtained by the study of three partial reactions that are catalyzed by CPS (Anderson & Meister, 1966) and the use of positional isotope-exchange experiments (Raushel & Villafranca, 1979, 1980). The three partial reactions that CPS catalyzes are: (1) the bicarbonate-dependent hydrolysis of ATP; (2) ATP synthesis from ADP and carbamoyl phosphate; and (3) hydrolysis of glutamine to glutamate and ammonia (Wellner, Anderson & Meister, 1973).

Carbamoyl phosphate synthetase is a heterodimer of approximately 159 kDa consisting of two non-identical subunits: a 41 kDa glutaminase subunit, and the 118 kDa synthetase subunit. The glutaminase subunit possesses the catalytic glutamine amide-N transfer domain, whereas the synthetase subunit catalyzes the formation of carbamoyl phosphate (Piette *et al.*, 1984; Nyunoya & Lusty, 1983). The latter subunit also contains binding sites for the allosteric effectors inosine 5' monophosphate (IMP), uridine 5' monophosphate (UMP) and ornithine (Anderson & Meister, 1966; Trotta, Burt, Haschemeyer & Meister, 1971). There is much evidence that indicates that there are two physically distinct ATP-binding sites in the larger subunit (Powers, Griffith & Meister, 1977; Powers & Meister, 1978a,b; Raushel *et al*, 1978; Raushel & Villafranca, 1979; Boettcher & Meister, 1980; Lusty, Widgren, Broglie & Nyunoya, 1983; Post, Post & Raushel, 1990). Comparison of the sequence of the synthetase subunit reveals that the amino-terminal and carboxyl-terminal halves show a high degree of internal homology. This has been suggested to have arisen from a gene duplication and fusion of an ancestral protein during the evolution of this enzyme (Nyunoya & Lusty, 1983). The homology in the primary structures between these two halves of the large subunit suggests that each domain may contain a separate binding site for one of the two ATP molecules required for carbamoyl phosphate synthesis.

In addition to the very large number of substrates this enzyme binds, it is also allosterically regulated. In the presence of positive allosteric effectors such as ornithine, IMP, ammonia or MgATP the enzyme oligomerizes into a tetramer. Conversely, removal of potassium ions or addition of negative effectors such as UMP favors formation of the monomer (Trotta *et al.*, 1971; Powers & Meister, 1978b; Anderson, 1977; Powers, Meister & Haschemeyer, 1980; Boettcher & Meister, 1982). Given the biological importance of this enzyme and its unusual enzymatic and structural organization an X-ray crystallographic study has been initiated. Crystals of carbamoyl phosphate synthetase are reported here that are suitable for a detailed structural analysis.

Materials and methods

Materials

HEPES and HEPPS were from Research Organics, Inc. (Cleveland, OH). KOH and KCl were from Mallinckrodt

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(Paris, KY). PEG 8000, L-ornithine, $MnCl_2$, NEt_4Cl, and ethylene glycol (spectrophotometric grade) were from Aldrich (Milwaukee, WI). NaN_3 and ADP [as the di(monocyclohexyl-ammonium) salt] were from Sigma (St Louis. MO). All other materials were from the references noted.

Crystal screening procedures

These were initially carried out with hanging-drop vapor-diffusion experiments. The enzyme was purified from *E. coli* RR1 carrying the plasmid pDP412 (Mullins, Lusty & Raushel, 1991). For crystallization experiments the purified CPS was dialyzed twice at 277 K against 2 l of 10 mM HEPES (pH 7) containing 100 mM KCl and 0.5 mM NaN_3. Following dialysis the protein was concentrated as needed with a Centricon 30 (Amicon). Initial precipitation points were identified using precipitants that included PEG, sodium/potassium phosphate, ammonium sulfate, lithium sulfate, sodium citrate and sodium formate over a pH range 5.0–8.0. The most promising crystals were obtained from PEG at 277 K. Attempts to improve crystal morphology and size included the use of various effectors and substrate analogs (L-ornithine, ATP, ADP, UMP, UDP, GTP, UTP, IMP, N-acetyl-L-glutamate, 6-azido-5-oxo-L-norlucine and azaserine) and a various array of cations and anions (K^+, Na^+, NMe^{4+}, NEt^{4+}, Li^+, Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, NH^{4+}, Cl^-, Br^-, HCO^{3-}, SO_4^{2-}, $C_6H_5O_7^{3-}$ and HCO^{2-}) in combination with PEG 8000 and 10000. The best crystals were grown from PEG 8000 and NEt_4Cl in the presence of KCl, L-ornithine, $MnCl_2$ and ADP.

Conditions were optimized such that large single crystals of CPS (Fig. 1) could be grown from batch experiments at 277 K in the presence of 100 mM KCl, 0.5 mM L-ornithine, 0.5 mM $MnCl_2$, 2.5 mM ADP, with 8% PEG 8000 and 0.65 M tetraethylammonium chloride buffered at pH 8 with 12.5 mM HEPPS. To 445 µl of dialyzed CPS at 9 mg ml^{-1} were added (with gentle vortexing) 5 µl of a freshly prepared solution containing 100 mM L-ornithine and 100 mM $MnCl_2$ and 50 ml of a freshly prepared 50 mM solution of ADP. To this solution was then slowly added 500 µl of a solution containing 16% PEG 8000, 1.3 M tetraethylammonium chloride and 100 mM KCl that had been buffered at pH 8 using 25 mM HEPPS. Gentle vortexing was used to thoroughly mix the protein solution with the precipitants. The fully mixed solution was then microfuged for 2 min to remove any precipitated materials. 100 µl aliquots of this solution were then placed in the wells of a Plexiglas
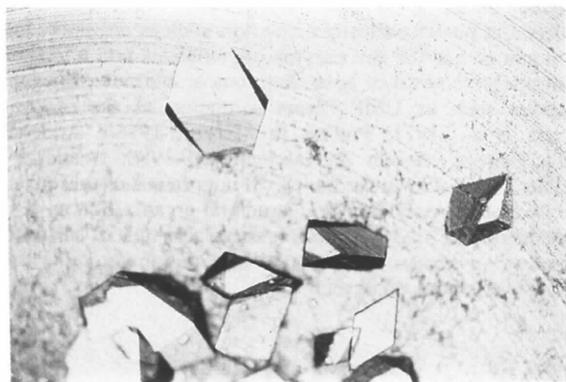
depression tray that had been lightly lubricated with silicon oil. The wells were then covered with glass coverslips and sealed with silicon oil to prevent evaporation. The diamond-shaped crystals, which generally appeared within 1 to 2 weeks, could reach a maximum size of about $1 \times 0.5 \times 0.4$ mm over the course of 4 to 10 weeks.

Crystal manipulation and X-ray diffraction

The initial characterization was performed by wet-mounting the crystals in thin-walled quartz capillary tubes (Charles Supper Co.). This was carried out by leaving as large a puddle of mother liquor around the crystal in the capillary tube as possible without leaving so much that the crystal would slip. An additional drop of mother liquor was left a short distance (1–2 mm) from the crystal on either side in the capillary tube. The ends of the capillary were then sealed with silicon oil (placed so as to almost touch the extra drops of mother liquor) and finally with wax. Still-setting and small-angle ($\mu = 2°$) precession photographs were recorded using nickel-filtered Cu $K\alpha$ radiation from a Rigaku RU200 rotating-anode X-ray generator with a 200 mm focal spot operated at 50 kV and 50 mA that was equipped with double focusing mirrors. A crystal-to-film distance of 10 cm was used, and the crystals were maintained at 277 K using a stream of cooled air. Once it was ascertained that the crystals diffract well, but were radiation sensitive, conditions for flash-cooling were established. These latter experiments are described below.

Results and discussion

Small-angle precession photographs of the 0kl and h0l zones showed that the crystals apparently belonged to the orthorhombic space group $P2_12_12_1$ with unit-cell dimensions of $a = 154.4$, $b = 166.5$ and $c = 338.7$ Å. Still setting photographs showed diffraction to approximately 2.8 Å resolution. A 1° rotation photograph (Fig. 2) recorded at SSRL with an MAR scanner at a wavelength of 1.08 Å displayed good diffraction to the edge of the image (2.7 Å resolution). Assuming a

Fig. 1. Typical crystals of carbamoyl phosphate synthetase grown in the presence of L-ornithine, $MnCl_2$, KCl, and ADP. Growth occurred at 277 K with PEG 8000 and NEt_4Cl as precipitants
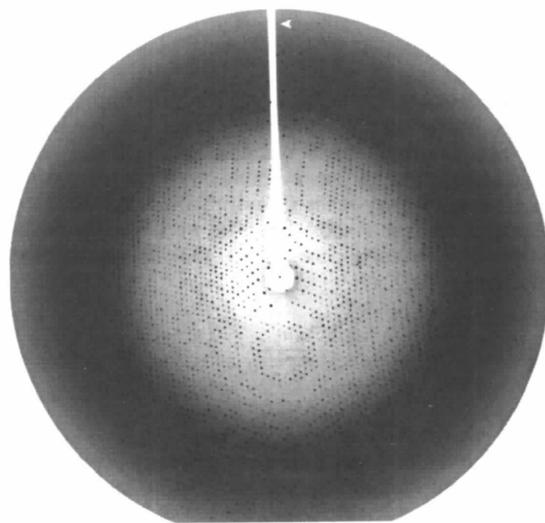

Fig. 2. A 1° rotation photograph taken at SSRL with a MAR scanner at a crystal-to-plate distance of 360 mm. The arrow on the diffraction pattern indicates a reflection at 2.85 Å resolution.

molecular mass of approximately 159 kDa and one tetramer per asymmetric unit, the V_m value is 3.4 Å^3 Da^{-1}, which lies within the range (1.68–3.53 Å^3 Da^{-1}) observed for globular proteins (Matthews, 1968). The crystals were relatively sensitive to radiation damage and diffracted strongly for only ∼12 h with radiation from a rotating-anode X-ray generator.

The majority of crystals exhibit a diamond-shaped morphology, however approximately 20% of the crystals possess a different morphology. These crystals have a tetragonal bipyramidal shape and represent an alternative crystal form. The lattice parameters of these crystals are roughly 195 ×.220 × 415 Å, and they belong to a C-centered orthorhombic space group. The diffraction quality of this crystal form is similar to that of the major form. Given the larger unit cell of this second form, efforts have focused on the crystals that exhibit the primitive orthorhombic lattice.

Native data to 3.5 Å resolution were collected at 277 Å on a Siemens X1000 area detector equipped with a helium path and double focussing mirrors. 0.15° frames were collected at a crystal-to-detector distance of 29 cm with an exposure time of 180 s frame^{-1}. The data were processed with the data-reduction software package XDS (Kabsch, 1988a,b), and scaled with XSCALIBRE (Wesenberg & Rayment, 1995). A total of 28 2310 reflections were collected from eight crystals which reduced to 88 121 of 110 466 possible independent reflections with an R_{merge} of 7.4% (where $R_{merge} = \Sigma|I_{hi} - I_h|/\Sigma I_{hi} \times 100$ and I_{hi} and I_h are the intensities of the individual and mean structure factors). Systematic absences observed in the $h0l$, $hk0$, and $0kl$ data were, to the limit of the data presently collected, in accordance with $P2_12_12_1$ space-group symmetry. The quality of this low-resolution data was acceptable for a cell of this magnitude, however it was clear that collection of high-resolution data would require a large number of crystals that might reduce the accuracy of the subsequent data and structure analysis. For this reason, conditions for flash-cooling the crystal have been established as described below.

Prior to flash-cooling the crystals, ethylene glycol was introduced into the mother liquor as a cryoprotectant. Initially it was observed that serial transfer of the crystals into solutions of increasing ethylene glycol concentration invariably caused the crystals to shatter. This problem was overcome by increasing the NEt$_4$Cl and KCl concentrations while the ethylene glycol concentration was also being raised. The crystals are transferred through five steps over a period of 1.5 h during which time the ethylene glycol concentration is linearly stepped up to 15% while the concentration of NEt$_4$Cl and KCl are linearly stepped to 1.0 M and 245 mM, respectively. The transferred crystals were then mounted using loops of fine surgical thread and flash-cooled to 108 K with a Siemens LT-2 low-temperature device. Still-setting photographs of the flash-cooled crystals showed diffraction to between 2.7 and 2.5 Å resolution with radiation from a rotating-anode X-ray generator and very little increase in the mosaic spread. In addition the crystals showed minimal loss of diffraction intensity after prolonged exposure to X-rays. The diffraction properties of the frozen crystals were superior to those that were wet-mounted. An initial determination of approximate lattice lengths at 108 K showed a 1.5–3% shrinkage from their values at 277 K.

A search for isomorphous derivatives is in progress. Several low-resolution data sets were collected at 277 K on crystals soaked in several platinum, mercury and lead compounds. The platinum- and mercury-treated crystals showed small changes. The crystals soaked with trimethyllead acetate showed larger changes, but these crystals proved to be much more radiation sensitive than the native crystals. Now that a protocol for flash-cooling the crystals has been developed, this problem should be overcome. Additional derivatives will be searched for with flash-cooled crystals.

Determination of the structure of carbamoyl phosphate synthetase at 2.7 Å resolution should reveal the relationships between the active sites and account for the allosteric behavior of this molecule. In addition, it will establish a structural basis for a continued study of this protein by site-directed mutagenesis.

Note added in proof: refined structures of the RC's from two other *Rb. sphaeroides* strains were recently published (Chirino *et al.*, 1994; Ermler, Fritzsch, Buchanan & Michel, 1994).

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