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Molecular Size and Symmetry of *Pseudomonas aeruginosa* Catabolic Ornithine Carbamoyltransferase

An X-ray Crystallography Analysis

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The catabolic ornithine carbamoyltransferase (EC 2.1.3.3) from *Pseudomonas aeruginosa*, that shows allosteric behaviour, and a mutant version of this enzyme has been crystallized in several different crystal forms. All of these have been characterized by X-ray diffraction methods. A 4·5 Å resolution data set has been collected on a triclinic crystal. Analysis of the data using the self-rotation function shows that 12 monomers associate to form a particle with cubic 23 point group symmetry.

Keywords: carbamoyltransferase; ornithine; oligomer; allostery; crystallization

Pseudomonas aeruginosa has two ornithine carbamoyltransferases (OTCase§; E.C. 2.1.3.3.; Stalon *et al.*, 1967; Haas *et al.*, 1979; Stalon & Mercenier, 1984). An anabolic enzyme participates in arginine biosynthesis and catalyses the formation of citrulline and phosphate from ornithine and carbamoylphosphate. In keeping with several anabolic OTCases from various organisms, that from *Ps. aeruginosa* is a trimer consisting of equal subunits of 37 kDa (Itoh *et al.*, 1988). A catabolic enzyme is involved in the anaerobic degradation of arginine *via* the arginine deiminase pathway (Stalon & Mercenier, 1984; Vander Wauven *et al.*, 1984) and catalyses the reverse reaction, the phosphorolysis of citrulline to produce ornithine and carbamoylphosphate. It is this catabolic form of the enzyme which is the subject of the present paper. The catabolic OTCase of *Ps. aeruginosa* is built up by the polymerization of trimers of identical subunits (38 kDa) into an active nonamer or a dodecamer (Baur *et al.*, 1987). Among the 11 OTCases whose sequences are known, only that from *Ps. aeruginosa* fulfils a catabolic function, and moreover uses homotropic and heterotropic interactions to regulate its activity. The equilibrium of the catalysed reaction strongly favours citrulline synthesis (Stalon *et al.*, 1977). However, the catabolic enzyme cannot perform citrulline synthesis *in vivo* because of its extreme cooperativity with respect to carbamoylphosphate (Haas *et al.*, 1979, 1989). The native enzyme also displays sigmoidal kinetics with respect to citrulline and phosphate (C. Tricot, unpublished results). Phosphate, the product of the carbamoylation reaction, and nucleoside monophosphate act as activators increasing the affinity of the enzyme for carbamoylphosphate, whereas polyamines such as spermine, spermidine and putrescine alter the catalytic activity by decreasing its affinity for the substrate, carbamoylphosphate. The homotropic cooperative interactions between the catalytic sites have been explained by transition from a conformation of the enzyme which possesses low affinity for

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§ Abbreviations used: OTCase, ornithine carbamoyltransferase; DTT, dithiothreitol; PEG, polyethylene glycol.

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carbamoylphosphate (T state) to a conformation that has high affinity for this substrate (R state) according to the model of Monod *et al.* (1965). Single amino acid substitution mutants of OTCase have been produced (Haas *et al.*, 1979, 1989). The substitution of glutamate for glycine at position 106 in the polypeptide chain alters the properties of the enzyme. The E106G-substituted mutant has almost lost the homotropic co-operativity for carbamoylphosphate observed in the wild-type enzyme with a concomitant increase in affinity for this substrate and in specific activity, allowing its utilization *in vivo* for citrulline synthesis (Haas *et al.*, 1979, 1989). The mutant enzyme has the same molecular weight and, probably, the same quaternary structure as the wild-type enzyme.

To address the question of the mechanism of the allosteric transition of OTCase and, in particular, the propagation of indirect interactions between the catalytic and regulatory sites, we performed crystallographic experiments in which OTCases (wild-type and mutant versions) were both crystallized with a combination of products or effectors. We report here the growth of four crystal forms obtained in hanging drops, using ammonium sulphate and polyethylene glycol (PEG 6000) as precipitant. The crystallization of *Escherichia coli* anabolic ornithine OTCase has been published (Kuo & Seaton, 1989). In these crystals the active trimer is present in the asymmetric unit. The quaternary structure of this enzyme is different from that of the catabolic enzyme, both in solution and in the crystal.

The wild-type and the E106G mutant enzymes were genetically overexpressed in *Ps. aeruginosa*, and purified by a modification of the method described by Baur *et al.* (1987). The two last steps were replaced by an arginine-Sepharose column (Pharmacia, 2·5 mm × 35 mm) equilibrated with 10 mM-potassium phosphate buffer (pH 7·5). The column was washed with the same buffer until the absorbance of the eluant at 280 nm was zero. The enzyme was then eluted by a linear gradient of 0 to 0·2 M-KCl in 10 mM-potassium phosphate (pH 7·5). The enzyme was homogeneous when examined by polyacrylamide gel electrophoresis run under denaturing and non-denaturing conditions. A typical 2·0 litre bacterial cell culture yielded 85 mg of purified native catabolic OTCase and 120 mg of the purified mutant enzyme.

Concentrated protein samples (15 to 26 mg/ml) were prepared in various buffers, containing 1 mM-dithiothreitol (DTT) and 1 mM-EDTA. They were mixed with known volumes of the precipitating agent diluted in the same buffer. Droplets (10 to 16 μl) were suspended from the underside of microscope cover slips siliconized with 2% (v/v) dimethyldichlorosilane in 1,1,1-trichloroethane, which were then placed over a 1-ml well containing the undiluted precipitating agent. All the crystallization boxes were kept at 20 °C. For the first form obtained, crystals grew as dome-like objects, 0·2 to 0·4 mm in each dimension. Sometimes additional larger prismatic crystals appeared in the same drops. Various conditions were tested in order to stabilize these forms. Finally, we were able to produce large crystals suitable for X-ray analysis. Crystals were mounted and sealed in capillary tubes. Unit cell dimensions and space group were determined using an Enraf-Nonius precession camera. Cell parameters of the hexagonal form were refined on a Siemens diffractometer. For each form, the crystal density was measured in the manner described by Colman & Matthews (1971). The results are summarized in Table 1.

Form D crystals were used to solve the symmetry of the oligomer. X-ray diffraction data were

Table 1

Crystallographic data of four OTCase crystal forms

Form	A	B	C	D
Crystallization conditions	Hepes 50 mM DTT 1 mM EDTA 1 mM Spermidine 10 mM pH 7·2	Cacodylate 50 mM DTT 1 mM EDTA 1 mM pH 6·2	Hepes 50 mM DTT 1 mM EDTA 1 mM pH 7·6	PO_4^{3-} (K^+) 50 mM DTT 1 mM EDTA 1 mM Ornithine 20 mM pH 7·5
Precipitating agent	$(NH_4)_2SO_4$ 47% of saturation	PEG 6000 13% (w/v) MPD 10% (v/v)†	$(NH_4)_2SO_4$ 50 to 54% of saturation	$(NH_4)_2SO_4$ 47 and 50% of saturation
Unit cell	a = 141 Å b = 141 Å c = 145 Å γ = 120°	a = 179 Å b = 179 Å c = 120 Å γ = 120°	a = 126 Å b = 133 Å c = 134 Å α = 61° β = 50° γ = 58°	a = 130·15 Å b = 129·65 Å c = 133·22 Å α = 60·83° β = 60·74° γ = 60·11°
Space group	$P6_3$	$R3$	$P1$	$P1$
Measured density	1·24 g/cm^3	1·14 g/cm^3	1·22 g/cm^3	1·23 g/cm^3
Protein	Wild-type (wt)	wt	E106G	wt

† MPD, 2-Methyl-2,4-pentanediol.

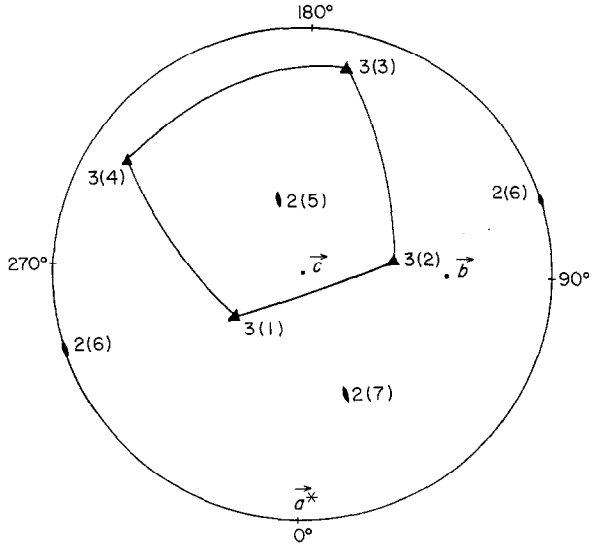

Figure 1. Self-rotation function of *Ps. aeruginosa* catabolic OTCase. Stereographic projection of the main peaks for $\kappa = 120°$ and 180° are shown. The location of the crystallographic axes $\vec{a}^*$, $\vec{b}$, and $\vec{c}$ are indicated. Peak numbers of Table 2 are in parentheses.

collected using CuKα monochromatized radiation from a RIGAKU RU200 rotating anode generator and a Siemens area detector. The native data set was recorded in 1210 frames of 0·2°, in two different orientations of the crystal. The observations were reduced to 32,268 unique reflections with an R_{merge} value ($\Sigma\Delta I_i/\Sigma I_i$) of 0·05. The data set was 77% complete to 4·5 Å (1 Å = 0·1 nm).

A self-rotation function (Rossmann & Brow, 1962) was calculated with the computing package MERLOT (Fitzgerald, 1988) using the data between 12 Å and 9 Å resolution and a radius of integration of 53 Å. The main features of the scaled map are: 0·15, 100, 5·97, for the average, maximum and root-mean-square values, respectively. Significant peaks (greater than 10 root-mean-square above the mean value) are located in the sections $\kappa = 180°$ and 120° (Table 2). The stereographic projection (Fig. 1) shows clearly the relative orientation of the 3-fold and 2-fold axes. The angles between all these axes are close to the theoretical values calculated for a 23 point group symmetry (3–3: 70·52°, 3–2: 54·73°,

Table 2
Data from the self-rotation function

Peak number	Psi (°)	Phi (°)	Kappa (°)	Height	Next highest peak
1	35	305	120	100	
2	40	100	120	75	
3	85	170	120	77	
4	80	240	120	84	48
5	35	200	180	77	
6	90	290	180	75	
7	55	20	180	75	

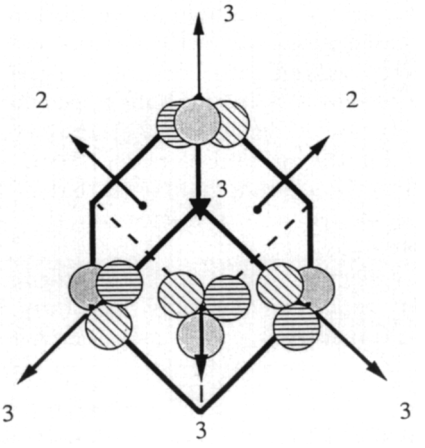

Figure 2. Symmetry of the oligomer; 2 and 3 are 2- and 3-fold rotation axes, respectively. Two-fold-related monomers have the same shading.

2–2: 90°) (Fig. 2). Taking into account the molecular mass, the oligomer is composed of 12 subunits. Quite interestingly the only known example of such high symmetry in an oligomeric enzyme has also been observed in *Ps. aeruginosa* for protocatechuate 3,4-dioxygenase (Satyshur *et al.*, 1980). Moreover, for this enzyme the $\alpha_2\beta_2$ tetramers associate to form a particle with the 23 point group symmetry. The structure has been solved to 3 Å resolution (Ohlendorf *et al.*, 1988).

The protein content parameters V_m (Matthews, 1968) have been estimated for each crystal form based on the unit cell volume, the molecular mass of 38,013 daltons for a monomer and symmetry elements in common between the oligomer and the unit cell (Table 3). These values are within the range of 1·68 to 3·53 Å^3/dalton commonly observed in crystalline proteins. Higher V_m values for the two triclinic forms suggest that the molecular packing is more tenuous. For these two forms also, the three crystallographic axes are almost identical (~130 Å) and the three angles are close to 60°. This pseudo-rhombohedral cell is also a pseudo cubic one (a_c = 184 Å) with a face-centred lattice (F). In this cubic unit cell, a close packing of spheres with a radius of 65 Å is possible, suggesting a more-or-less spherical

Table 3
Number of oligomers in the unit cell

Form	A	B	C	D
Space group	$P6_3$	$R3$	$P1$	$P1$
Number of oligomers per cell	2	3	1	1
V_m (Å^3/dalton)	2·73	2·40	3·11	3·51
Symmetry element in common	3	3	1	1
Allosteric state	?	T	R	R

oligomer particle. In Table 3, we also report the expected allosteric state of the enzyme which would be expected taking into account the pH and the presence of effectors in the buffer used for crystallization. It should be noted that the crystal form obtained for the mutant version E106G in Hepes buffer (form C) appears similar to that of the wild-type enzyme in the presence of the activator phosphate.

Data collection on the other forms and a molecular replacement study on form D using aspartate transcarbamoylase as a search model are underway.

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