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Crystallization and preliminary crystallographic analysis of creatininase from *Pseudomonas putida*

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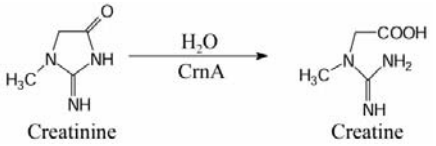
Creatininase (CrnA) from *Pseudomonas putida* is a homohexameric heat-stable enzyme composed of 259 amino acids per subunit. The molecular weight of each monomer is 28.4 kDa. The enzyme hydrolyses creatinine to yield creatine. Crystals of this protein have been grown from ethanol/PEG 8000. They belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 74.8, b = 95.7, c = 116.9 Å, $\alpha = \gamma = 90$, $\beta = 103.8^\circ$. The diffraction limit is 2.5 Å. The self-rotation function of the native data set is consistent with a CrnA hexamer in the asymmetric unit and suggests D_3 point-group symmetry of the enzyme.

1. Introduction

Creatininase (CrnA), an enzyme which so far has been found in several bacterial species such as *Pseudomonas* and *Alcaligenes* (Wyss & Kaddurah-Daouk, 2000), catalyzes the cleavage of the cyclic amide bond of creatinine to creatine.



Creatinine Creatine

In further degradation steps, creatine is hydrolysed to sarcosine and urea by creatinase and finally demethylated by sarcosine oxidase to yield glycine. Organisms which possess creatininase activity can use creatinine as a carbon and nitrogen source.

In addition to this metabolic pathway, another degradation path has been described starting with CrnA. In this pathway, creatinine is hydrolysed by *N*-methylhydantoinase to yield *N*-methylhydantoin, which is further hydrolysed to *N*-carbamoylsarcosine in the presence of ATP by *N*-carbamoylsarcosine amidohydrolase. The regulation and biochemical function of these two alternative pathways is still unknown.

CrnA plays an important role in diagnostics as it can serve to create enzymatic biosensors to determine the concentration of creatinine.

In mammals, creatinine preferentially occurs in muscle cells as a metabolic end product which does not undergo further degradation steps. It is glomerularly filtrated in kidney and is excreted with the urine.

Creatinine is produced by the non-enzymatic cyclization reaction of creatine and creatine phosphate. Creatine is synthesized in two biosynthetic steps. The transfer of the amidino group of L-arginine to glycine to yield L-ornithine and guanidinoacetic acid represents the first of the two steps in the biosynthesis of creatine and is catalyzed by L-arginine-glycine amidinotransferase. Guanidinoacetic acid is then methylated at the amidino group to give creatine. This reaction step is catalyzed by *S*-adenosyl-L-methionine-*N*-guanidinoacetate methyltransferase. The first step of creatine biosynthesis occurs mainly in the kidney, whereas the methylation of guanidinoacetic acid to creatine takes place in the liver (Wyss & Kaddurah-Daouk, 2000). Creatine then diffuses to muscle cells, where creatine phosphate is generated by creatine kinase with consumption of ATP.

As creatinine is proportional to muscle mass and only glomerularly filtrated, its concentration in blood, serum and urine gives valuable information about renal function.

Sequence comparisons showed homology to other CrnAs in bacteria and archaea, but no homology to other enzymes could be found. Furthermore, based on the sequence there were no homologous structures in the PDB. Therefore, the CrnA structure probably differs from other amidohydrolase structures. Because of this, it might be interesting to determine the structural differences from dihydropyrimidinases, allantoinases and dihydroorotases (Wilcox, 1996; Thoden *et al.*, 2001), which also act on a cyclic amide bond in their substrates. All enzymes mentioned above possess a metal centre; like these enzymes, CrnA is described as being a zinc metallo-

enzyme (Rikitake *et al.*, 1979). Structural analysis of CrnA will thus provide insights into the structure of this putative novel metal centre.

2. Materials and methods

2.1. Protein preparation

The CrnA gene of *P. putida* was cloned and expressed in *Eschericha coli* by Roche Diagnostics, Penzberg, Germany (unpublished results). The enzyme was purified by an initial heat separation step followed by several chromatographic separation steps (Roche Diagnostics, Penzberg, Germany, unpublished results).

Samples for this study had a purity of >95% as judged by SDS–PAGE. MALDI mass spectrometry gave a molecular mass of 28.4 kDa for one subunit. This is in good agreement with the theoretical molecular mass of 28 399 Da. Dynamic light-scattering analysis showed a monomodal size distribution with an apparent molecular weight of 173 kDa, which corresponds to the mass of the hexamer. This quaternary structure was also confirmed by gel filtration. Prior to crystallization, the protein was concentrated and dialyzed against 20 m*M* Tris pH 8.0 by ultrafiltration (Amicon, Centricon). The final concentration of the protein was determined by UV–Vis absorbance spectroscopy at 520 nm against BSA as an internal standard using the Bradford procedure (BioRad, Microassay; Bradford, 1976).

2.2. Crystallization

Initial crystallization experiments were based on the sparse-matrix sampling method (Jancarik & Kim, 1991). Crystals of CrnA could be obtained at pH 6.5 by the sitting-drop variant of the vapour-diffusion method at 293 K in the presence of NaOAc/PEG 8000 and $Mg(OAc)_2$/PEG 6000. Unfortunately, crystals grown under these conditions were mostly poorly shaped and difficult to reproduce. However, these initial crystallization trials were valuable because they showed a pH dependence of the enzyme precipitation. The enzyme tends to precipitate at pH values of under 6.0 and over 9.0, corresponding to the stability range of the enzyme (Rikitake *et al.*, 1979).

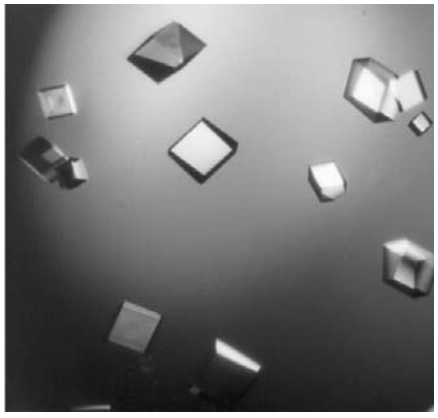


Figure 1
Typical CrnA crystals of dimensions 0.3 × 0.3 × 0.2 mm.

2.3. Optimization of crystallization

Further crystallization experiments were based on the results of dynamic light-scattering experiments. It was found that CrnA tends to aggregate in the presence of several salts commonly used as precipitants in protein crystallization protocols, such as ammonium sulfate, sodium acetate, calcium acetate and others (data not shown). On the other hand, there was a monomodal size distribution of the particles in solution with an apparent molecular weight of 351 kDa when using ethanol as precipitating agent. This might have been the reason for a cluster formation of two single CrnA hexamers in solution (Kam *et al.*, 1978; D'Arcy, 1994). Therefore, crystallization trials were performed with different concentrations of ethanol combined with PEGs of different sizes in the pH range 6–9.

Crystals were grown using the vapour-diffusion method with a sitting drop at 293 K consisting of 3 µl CrnA (12 mg ml^{-1}) and 3 µl of reservoir solution containing 22% ethanol, 20% PEG 8000, 0.1 *M* MOPS pH 7.5 equilibrated against 1000 µl of the reservoir solution.

2.4. Data collection and analysis

A native data set was collected on a rotating copper anode generator (Nonius) operating at 45 kV and 100 mA with a MacScience DIP 2030 detector at 100 K. The oscillation range per image was 1°. The crystals were transferred to cryoprotectant (crystallization buffer containing 15% glycerol) in a single step and placed directly into the nitrogen stream (Oxford Cryosystems Cryostream) for data collection. The data set was integrated and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

Table 1
Statistics of the native data set.

Data set	Native
Wavelength (Å)	1.54178
Resolution range (Å)	50–2.5
Space group	$P2_1$
No. of measured reflections	212677
No. of unique reflections	53895
Unit-cell volume ($Å^3$)	8.2×10^5
Completeness (%)	99.7 (99.3)
$I/\sigma(I)$	13.8 (2.8)
R_{sym} (%)	9.7 (45.2)

Self-rotation calculations were performed to search for non-crystallographic symmetry (NCS) using the fast rotation-function algorithm (Crowther, 1972) of the program *GLRF* (Tong & Rossmann, 1997). A native Patterson function was calculated using the program *FFT* from the *CCP*4 suite (Colla-

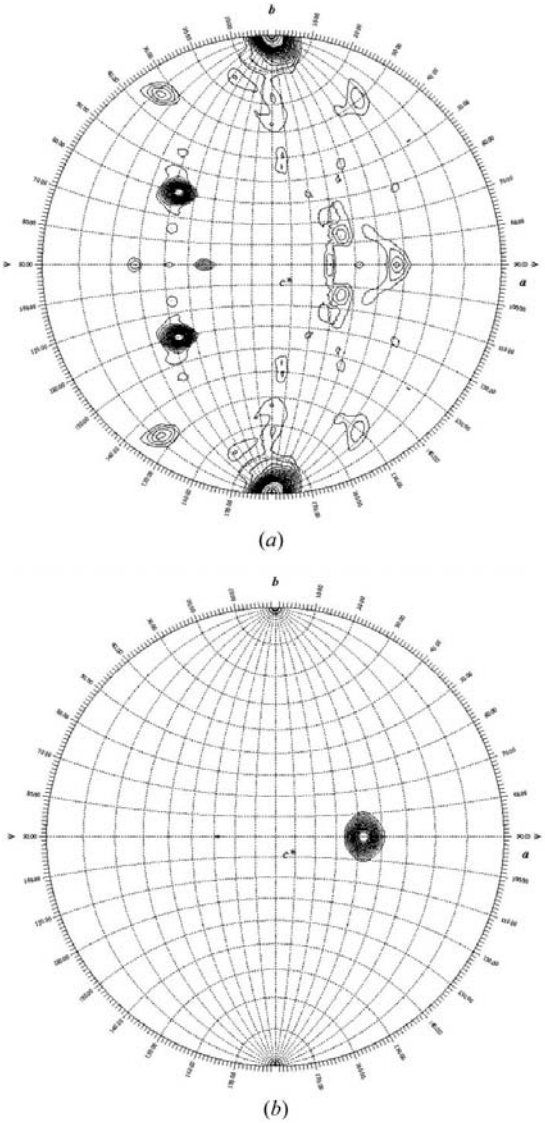


Figure 2
Self-rotation functions. (*a*) $\kappa = 180°$, (*b*) $\kappa = 120°$.

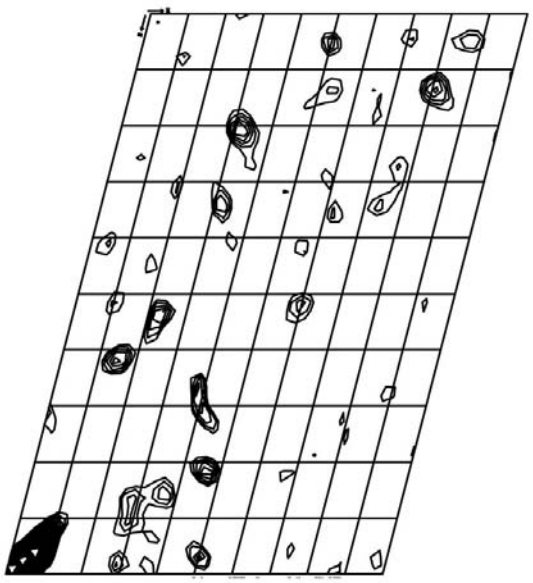


Figure 3
Native Patterson function (y = 0.5).

borative Computational Project, Number 4, 1994).

3. Results and discussion

Well shaped CrnA crystals were obtained within a few days. Typical dimensions of the crystals were 0.3 × 0.3 × 0.2 mm (Fig. 1). Diffraction patterns from the crystals were observed to a diffraction limit of 2.5 Å (Table 1). The space group was determined to be $P2_1$, with unit-cell parameters a = 74.8, b = 95.7, c = 116.9 Å, $\alpha = \gamma = 90$, $\beta = 103.8^\circ$. The $R_{\rm sym}$ value of the data set calculated over all resolution shells is 9.7%. The completeness of the data set between 50 and 2.5 Å is 99.7% and the redundancy of the data is 3.9.

The only possible $V_{\rm M}$ value that is within the usual range for protein crystals (Matthews, 1968) is 2.4 Å^3 Da^{-1}, corresponding to one hexamer in the asymmetric unit and a solvent content of 44%. The self-rotation function is consistent with a hexameric quaternary structure of the enzyme. It shows three peaks at $\kappa = 180^\circ$ and one peak at $\kappa = 120^\circ$ (Fig. 2). The peak at $\psi = 0$, $\varphi = 0^\circ$ corresponds to the crystallographic b axis. The two other large peaks at $\kappa = 180^\circ$ indicate local symmetry axes relating the subunits within the oligomeric protein molecule. A third twofold NCS axis should be parallel to the crystallographic b axis. The three twofolds are 60° distant from each other. The other peak at $\kappa = 120^\circ$ corresponds to a threefold axis perpendicular to the three twofolds. All these NCS peaks show intensities of 56 and 55% relative to the most intense peak. The self-rotation function confirms a hexameric quaternary structure and suggests D_3 point-group symmetry for the enzyme. The D_3 point-group symmetry means that CrnA is built as a dimer of trimers.

As one twofold NCS axis is supposed to be parallel to the crystallographic 2_1 axis, a significant peak at y = 0.5 is expected in a native Patterson function and is found. The calculated Patterson function shows an intensive ψ origin peak at (0.0, 0.5, 0.5). The height is 11% of the origin peak (Fig. 3).

As there were no structures homologous to CrnA in the PDB, phasing should be performed by MIR or MAD methods. The information from the self-rotation and the Patterson function should be valuable for finding heavy-atom derivatives, for molecular averaging and in other steps in the course of the phasing process.

A search for heavy-atom derivatives has been initiated and various platinum and mercury compounds have so far been investigated. When soaked overnight with 100 mM K_2PtCl_4 in artificial mother liquor, the crystals show a slight yellow colouring and remain well shaped. After preliminary crystallographic investigations, the crystals seem to be a derivative and might be suitable for a MAD experiment.

We gratefully acknowledge Roche Diagnostics, Penzberg, Germany for the gift of CrnA.

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