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Crystallization of ornithine acetyltransferase from yeast by counter-diffusion and preliminary X-ray study

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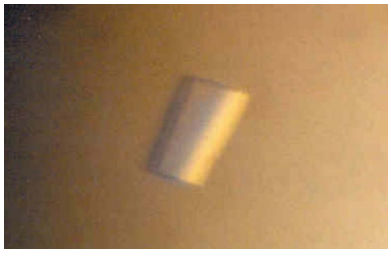


A study is presented on the crystallization of ornithine acetyltransferase from yeast, which catalyzes the fifth step in microbial arginine synthesis. The use of the counter-diffusion technique removes the disorder present in one dimension in crystals grown by either the batch or hanging-drop techniques. This makes the difference between useless crystals and crystals that allow successful determination of the structure of the protein. The crystals belong to space group $P4$, with unit-cell parameters $a = b = 66.98$, $c = 427.09$ Å, and a data set was collected to 2.76 Å.

1. Introduction

The development of crystallization techniques and strategies is based on two complementary approaches. Traditionally, trial-and-error methods are used. Simultaneously, a limited number of proteins have been the subject of more fundamental studies on crystallization processes (Giegé *et al.*, 1995; Vekilov & Chernov, 2002). Although in recent years the focus of structural genomics projects has been on high-throughput methods, there is still a need for a continued development of crystallization techniques and strategies that produce better quality crystals.

One of the approaches used to obtain crystals of high quality is the use of counter-diffusion techniques (García-Ruiz, 2003). The success of the method depends on the occurrence of a supersaturation wave that travels through the protein chamber with the crest becoming lower, enabling the simultaneous screening of a variety of conditions (García-Ruiz *et al.*, 2001). Counter-diffusion techniques have led to the production of crystals of increased perfection for various proteins that have been tested (Maes *et al.*, 2004; Ng *et al.*, 2003). A prerequisite for the use of this technique is that mass transport must be controlled by diffusion alone. This can be achieved by working in reduced gravity, in gels or in microfluidics systems. Crystallization in microgravity has been shown to lead to better quality crystals in a significant number of cases, but can also result in inferior crystals (McPherson, 1993; Snell & Helliwell, 2005; Vekilov, 1999; Vergara *et al.*, 2003). The effect of microgravity on the perfection of crystals has been attributed to the formation of a protein-depletion zone around growing crystals (Rosenberger, 1986; McPherson, 1993), to the differential incorporation of impurities in nonconvective environments (Thomas *et al.*, 2000) and to the reduction of kinetic fluctuations at the growth surface of the crystals (Vekilov, 1999). The use of gels to mimic microgravity conditions or diffusive regimes enables the use of the counter-diffusion technique on earth (García-Ruiz, 2003).

Here, we present a study on the crystallization of ornithine acetyltransferase (OAT) from yeast. OAT is a key enzyme in microbial arginine biosynthesis and has a molecular weight of 47 849 Da (Crabeel *et al.*, 1997; Sakanyan *et al.*, 1993). It transfers the acetyl group of *N*-acetylornithine to glutamate, thereby forming ornithine, while regenerating *N*-acetylglutamate (NAG). OAT is coded by the *argJ* gene in bacteria and by *ARG7* in the yeast *Saccharomyces cerevisiae*. Although all characterized OAT enzymes are coded by a single open reading frame, they have been shown to be composed of two distinct subunit peptides α and β (Liu *et al.*, 1995). As was first proposed for the yeast OAT enzyme Arg7p, the α and β subunits are generated from the precursor protein by an auto-

Table 1
Crystallization conditions.

In a convective environment
Hanging drop
Protein solution: 20 m*M* Tris–HCl pH 7.6, 100 m*M* NaCl, 0.5 m*M* EDTA
Protein concentration: 6.5 mg ml^{-1}
Precipitants
(1) 20% MPD in 20 m*M* Tris–HCl pH 7.0
(2) 22% ethanol in 100 m*M* TEA pH 9.0
(3) 12% PEG 1500 in 100 m*M* trisodium citrate pH 5.6
(4) 10% PEG 4000 in 100 m*M* trisodium citrate pH 5.6
In a nonconvective environment (in agarose gels)
Batch
Protein solution: 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl + 0.1% agarose
Protein concentration: 5 mg ml^{-1}
Precipitant: 25–50% PEG 400 in 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl
Counter-diffusion in capillaries
Two-layer capillary counter-diffusion arrangement
Protein solution: 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl + 0.1% agarose
Protein concentration: 5 mg ml^{-1}
Precipitants
(1) 20–80% PEG 400 in 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl
(2) 20–80% MPD in 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl
Acupuncture method in GCB
Protein solution: 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl + 0.1% agarose
Protein concentration: 5.5 mg ml^{-1}
Precipitant: 60% PEG 400 in 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl

proteolytic cleavage between the invariant alanine and threonine residues located in a conserved KG*X*GM*XX*P*X*—(M/L)AT(L/M)L amino-acid sequence. This threonine, positioned at the N-terminus of a β-strand, is required for both self-cleavage and catalytic activity (Abadjieva *et al.*, 2000; Marc *et al.*, 2001). The α and β subunits have been shown to assemble as $\alpha_2\beta_2$ heterotetramers.

The first X-ray crystal structure of an OAT has recently been obtained to 2.8 Å resolution for the *orf6* gene product of *Streptomyces clavuligerus* (Elkins *et al.*, 2005; Kershaw *et al.*, 2002). This enzyme has a distinct OAT activity and is involved in the synthesis of clavulanic acid. Clavulanic acid is clinically used in combination with penicillin as a potent inhibitor of serine β-lactamases. The sequence identity with Arg7p is 29%.

The reported crystallization and data collection will aid us in solving the structure of a microbial OAT involved in arginine biosynthesis.

2. Material and methods

2.1. Purification of protein

Arg7p protein was expressed from vector pAA27 derived from pTrc99A in *Escherichia coli* BL21 (DE3) cells. The cells were grown at 303 K in 2×YT medium pH 7 supplemented with 0.7 g l^{-1} K_2HPO_4, 0.6 g l^{-1} KH_2PO_4 and 50 µg ml^{-1} ampicillin. When the culture reached an OD_{590} of 1, Arg7p synthesis was induced with 2 m*M* isopropyl β-D-thiogalactopyranoside (IPTG). Glucose was added to a final concentration of 1% and the culture was incubated for 6 h at 303 K. Cells were harvested by centrifugation at 4500*g* for 20 min, resuspended in 20 m*M* phosphate buffer pH 7.6, 10 m*M* $MgCl_2$, supplemented with EDTA-free protease-inhibitor cocktail tablets (Roche) and disintegrated with a high-pressure cell disrupter (Basic Z model, Constant Systems Ltd) at 20 MPa. The cell debris was removed by two rounds of centrifugation, first for 30 min at 10 000*g* and then for 1 h at 35 000*g*. The supernatant was filtrated through a 0.212 µm cellulose filter (Millipore) and NaCl was added to a final concentration of 250 m*M*. The supernatant was applied onto a Hi-Trap Chelating HP column (Amersham Bioscience) charged with Cu^{2+} and equilibrated with 20 m*M* Tris–HCl pH 7.6, 300 m*M* NaCl. The first washing step was performed with the same buffer in five column volumes. The second washing step was performed in 20 m*M* Tris–HCl pH 7.6, 20 m*M* imidazole, 500 m*M* NaCl for another five column volumes. A long gradient of imidazole was applied (to 50% in 30 column volumes) in which the protein was eluted between 15 and 20% imidazole, corresponding to 75–100 m*M*. The fractions containing Arg7p protein were gathered and dithiothreitol (DTT) was added to a final concentration of 2 m*M*. After a concentration step on Centriplus-50 (Amicon), a final polishing step was performed using the size-exclusion chromatography technique and a 12 HR 10/30 Superose column (Amersham Bioscience) equilibrated with 20 m*M* Tris–HCl pH 7.6, 250 m*M* NaCl, 2 m*M* DTT at a flow rate of 0.5 ml min^{-1}. The protein-rich fractions eluted as a single peak.

The protein was dialyzed against 20 m*M* Tris–HCl pH 7.6, 100 m*M* NaCl and 2.5 m*M* EDTA and concentrated to 6.5 mg ml^{-1}.

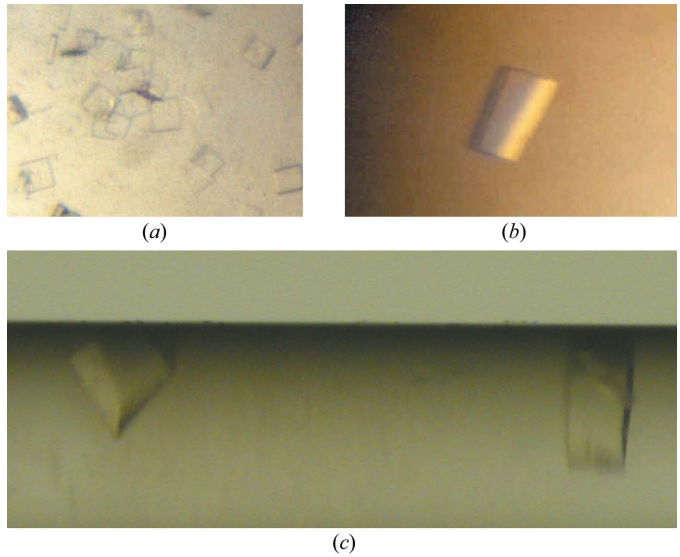
2.2. Dynamic light scattering (DLS)

The light-scattering experiments were carried out using a Zetasizer Nano S (Malvern Instruments) with a 633 nm He–Ne red laser. Prior to the scattering experiment, all solutions were filtered through a 0.22 µm filter (Millipore) and placed in a small-volume glass cuvette (Hellma). A series of 10 DLS measurements of 60 s each were performed at 293 K.

2.3. Crystallization

2.3.1. Crystallization in hanging drop. Crystallization trials were set up by the hanging-drop vapour-diffusion method at room temperature by sparse-matrix screening using commercial crystallization kits (Crystal Screen and Crystal Screen 2, Hampton Research, Riverside, CA, USA). 1–2 µl protein solution was mixed with an equal volume of reservoir precipitant solution and equilibrated against 0.5 ml reservoir solution. Plate-shaped crystals appeared in a variety of conditions within 2–4 d (Fig. 1*a*). After optimization of the conditions, the best looking crystals were obtained with a reservoir solution consisting of 20% MPD in Tris buffer pH 7.0, 22% ethanol in TEA buffer pH 9.0, 12% PEG 1500 in 100 m*M* trisodium citrate dihydrate pH 5.6 and 10% PEG 4000 in 100 m*M* trisodium citrate dihydrate pH 5.6 (see Table 1).

2.3.2. Crystallization in gels. Arg7p was crystallized in gel in three types of experiments: in batch, by capillary counter-diffusion in a two-



(*a*) (*b*) (*c*)

Figure 1
Crystals of Arg7p obtained (*a*, *b*) with the hanging-drop technique and (*c*) in gels.

layer arrangement and by the gel-acupuncture method in Granada Crystallization Boxes (GCBs; García-Ruiz *et al.*, 2002). A 1% agarose solution was prepared by adding 100 mg low-melting-point agarose to 10 ml crystallization buffer. The suspension was heated in a microwave oven until homogeneity and the solution was kept at 310 K. A protein solution was prepared by adding the 1% agarose solution to the protein solution in a 1:9 volume ratio.

Batch experiments were set up by mixing an equal volume of this gelled protein solution at 5 mg ml^{-1} with an equal volume of precipitant solution containing between 25 and 50% PEG 400 in crystallizing buffer (see Table 1).

In the two-layer capillary counter-diffusion experiment, the gelled protein solution was drawn up into a capillary, followed by an equal volume of precipitant solution. The precipitant solution consisted of crystallization buffer with either MPD (a range of 20–80% MPD) or PEG 400 (20–80%) (see Table 1). The capillaries, which had a thickness of 0.7 mm, were sealed at the top and bottom and were stored horizontally (Fig. 2).

The geometry of the acupuncture method in the GCB is shown in Fig. 2. The protein was brought to 5.5 mg ml^{-1} in the crystallization buffer (20 m*M* Tris–HCl pH 8, 0.1 *M* NaCl; see Table 1). The GCB was set upright and the 1% agarose solution was poured into the box until it reached a height of 1.5 cm. The gel set in 30 min at room temperature or 2–3 min at 253 K. After the gel had set, a thin layer of buffer was poured on top (100 µl). The protein solution containing 0.1% agarose was drawn into quartz capillaries (thickness 0.7 mm) by capillarity, keeping the capillary horizontal. The capillaries were sealed at the top with soft beeswax. The capillaries were then inserted into the GCB and pushed in the gel to an insertion depth of approximately 0.7 cm. A layer of precipitant solution (60% PEG 400, 20 m*M* Tris–HCl pH 8, 0.1 *M* NaCl) was poured on top until a height of 3 cm was obtained for the precipitant. The GCB was then sealed with vacuum grease and Scotch tape.

2.4. Data collection

X-ray diffraction data were collected at the DORIS storage ring (DESY, Hamburg) on beamline BW7A using a MAR CCD detector and on beamline BW7B using a MAR 345 detector (MAR Research, Norderstadt, Germany) at 100 K. No cryoprotectant was used. Crystals obtained by the various crystallization techniques were used. The final data set, collected from a single crystal obtained in a two-layer capillary counter-diffusion setup, was collected on BW7A. 40 images ($\Delta\varphi$ = 0.6) and 245 images ($\Delta\varphi$ = 0.4) were collected. The images were integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Purity of the sample

In order to determine the purity of the sample produced, we used SDS–PAGE with Coomassie blue staining. Two bands appeared corresponding to the α and β fragments, with molecular weights of 21 987 and 24 977 Da, respectively (Abadjieva *et al.*, 2000). Dynamic light scattering indicated a unimodal size distribution with a mean hydrodynamic radius value of 3.64 nm and a peak width of 0.67 nm, suggesting the presence of an Arg7p dimer ($\alpha_2\beta_2$).

3.2. Hanging-drop experiments

In first instance Arg7p crystallized very easily, producing platelet crystals (Fig. 1*a*). From diffraction experiments at synchrotrons (the ESRF in Grenoble and the DORIS storage ring in Hamburg), it became clear that the crystals were disordered in the orientation perpendicular to the plates. In order to overcome the problem of disorder, the crystallization conditions were first extensively optimized, changing the pH, precipitant, protein, precipitant concentration *etc*. This led to crystals that were no longer plates, but three-dimensional blocks (Fig. 1*b*).

When MPD was used as a precipitant, small well ordered crystals were obtained. It was possible to estimate the unit cell, but the diffraction limit was around 7 Å at beamline BW7A in Hamburg. A second problem with these crystals is the unit cell, which was estimated to have one axis of 450 Å.

3.3. Experiments in gel

In order to produce better quality crystals, experiments in a nonconvective environment (batch experiments in gels and counter-diffusion experiments) were set up (see Table 1).

Batch experiments were carried out at precipitant concentrations similar to or lower than those used in the hanging-drop setups, as nucleation and crystal growth in batch experiments is very rapid. This resulted in platelet crystals as with the hanging-drop method.

Counter-diffusion in capillaries can be very useful to optimize crystallization once initial conditions have been obtained for hanging-drop experiments. As the equilibration is achieved by diffusion only, the equilibration time tends to be much longer and is predominantly determined by the diffusion constant of the precipitant.

The conditions for the GCB counter-diffusion setups were derived from the hanging-drop experiments, taking into account the slower nucleation rates. We essentially kept the chemistry of the hanging-drop experiment, using the same buffer and pH. The higher-molecular-weight PEGs used as precipitant were replaced by PEG 400, as higher-molecular-weight PEGs have diffusion coefficients lower than 10^{-6} cm^2 s^{-1} (Vergara *et al.*, 1999). It takes months to fully equilibrate the precipitant in a GCB experiment (results not shown). Also, the precipitant concentration was chosen so as to obtain equilibrium concentrations that are much higher than those for hanging-drop experiments. The largest (0.2 mm) and best looking

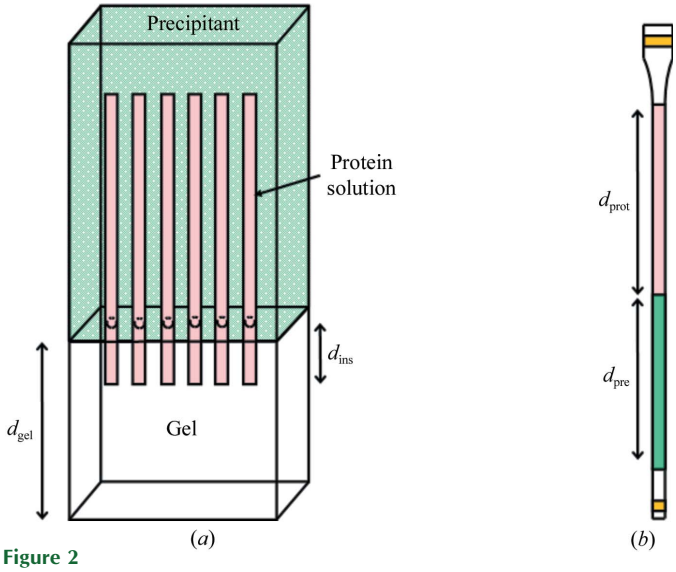


Figure 2
Counter-diffusion setups. (*a*) Granada Crystallization Box; (*b*) two-layer capillary counter-diffusion. d_{gel} = 2 cm, d_{ins} = 0.7 cm, d_{pre} = 2.5 cm and d_{prot} = 2.5 cm. Note that the protein solution contains a small percentage of gel.

Table 2
Data-collection statistics.

Values in parentheses correspond to the highest resolution shell.

Unit-cell parameters (Å, °)	$a = b = 66.98$, $c = 427.09$, $\alpha = \beta = \gamma = 90$
Space group	$P4$
Wavelength (Å)	0.9762
Resolution (Å)	35–2.8 (2.86–2.76)
Total no. of reflections	199903
No. of unique reflections	45012
Completeness (%)	93.5 (78.4)
Redundancy	4.4 (3.8)
Percentage of reflections with $I/\sigma(I) < 3$ (%)	11.2 (25.8)
R_{sym} (%)	14.4 (26.3)
Mosaicity (°)	0.409

crystals were obtained with a PEG 400 equilibrium concentration of 40%(*w*/*v*), whereas for hanging-drop experiments the best results were obtained with 20–25% PEG 4000.

Counter-diffusion experiments in capillaries were performed with final PEG 400 concentrations of up to 40%. In these experiments homogenization is faster than in GCBs and there is a direct contact between precipitant and protein solutions. This might explain the observation that nucleation was quite fast without using high precipitant concentrations. Crystals were obtained using PEG 400 and MPD as precipitants (Fig. 1*c*). The crystals are large (0.2 × 0.2 × 0.2 mm) and have clear faces.

4. X-ray data collection

Crystals obtained using the various crystallization techniques were used for X-ray diffraction. Only the crystals grown by counter-diffusion were of sufficient quality for collection of a data set. A data set was collected to 2.76 Å from a crystal obtained in a two-layer capillary counter-diffusion experiment using 70% PEG 400 as precipitant (∼35% final concentration). The crystal belongs to space group $P4$, with unit-cell parameters $a = b = 66.98$, $c = 427.09$ Å. The elongated unit cell hampered straightforward data collection and data processing. A typical diffraction image is given in Fig. 3. In order to keep the unit-cell parameters stable throughout the processing of the images, three-dimensional processing was used with *HKL*-2000. Data-collection parameters and statistics are summarized in Table 2.

In order to solve the structure, a selenomethionine derivative is being prepared. If necessary, bromide soaking will also be applied for phasing.

Figure 3
Diffraction image of a crystal obtained with a two-layer capillary counter-diffusion arrangement, with an enlargement shown as an inset.

5. Conclusion

We can conclude that in the case of Arg7p the use of the counter-diffusion technique was critical for the production of well ordered crystals, the first step in the structure-determination process.

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