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Expression, purification, crystallization and preliminary X-ray analysis of *Escherichia coli* argininosuccinate synthetase

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A recombinant form of *Escherichia coli* argininosuccinate synthetase with a C-terminal polyhistidine affinity tag has been expressed, purified and subsequently crystallized using the hanging-drop vapour-diffusion technique. The crystals grow as large rectangular chunks with unit-cell dimensions $a = 79.70$, $b = 105.84$, $c = 127.33$ Å, $\alpha = \beta = \gamma = 90^\circ$. The crystals exhibit the symmetry of space group $I222$ and diffract to a minimum d-spacing of 1.6 Å at station X8C of the National Synchrotron Light Source, Brookhaven National Laboratory. On the basis of density calculations, one monomer of this homotetrameric protein is predicted per asymmetric unit (Matthews coefficient $V_m = 2.69$ Å^3 Da^{-1}).

1. Introduction

Argininosuccinate synthetase (AS; E.C. 6.3.4.5) catalyzes the reversible ATP-dependent ligation of citrulline and aspartate to produce argininosuccinate, AMP and inorganic pyrophosphate (Fig. 1). The primary role of AS is in the urea cycle, which is responsible for the detoxification of ammonia in ureotelic species. Although this principal physiological role occurs in the liver and to a lesser extent in the small intestine, virtually all other mammalian tissues possess detectable levels of AS and a second urea-cycle enzyme, argininosuccinate lyase (AL; for reviews, see Jackson *et al.*, 1986; Meijer *et al.*, 1990). Together, these two enzymes have the net effect of producing arginine from citrulline and aspartate. In the kidney, AS and AL are responsible for the *de novo* synthesis of arginine to be released into the bloodstream (Windmueller & Spaeth, 1981). In the remaining tissues, however, the function of this metabolic route remained obscure until the discovery of arginine-derived nitric oxide (NO; Nathan, 1992). In many of these tissues, it has now been shown that the flavoprotein nitric oxide synthase (NOS) together with AS and AL produce NO. AS, AL and NOS constitute the arginine–citrulline cycle, an abbreviated urea cycle (Hattori *et al.*, 1994) that enables mammalian cells to sustainably overproduce NO, a key signaling molecule which has been found to elicit tumoricidal (Li *et al.*, 1991; Lorsbach *et al.*, 1993), antiviral (Karupiah *et al.*, 1993), bactericidal and fungistatic (Nathan & Hibbs, 1991) effects in the host defense system.

Owing to its role as a potent vasodilator, overproduction of NO is not entirely advantageous. Excess NO produced by the arginine–citrulline cycle is responsible for the hypotension of septic and cytokine-induced circulatory shock (Kilbourn, Gross *et al.*, 1990; Kilbourn, Jubran *et al.*, 1990). Septic shock is a life-threatening complication of bacterial infections which remains the major cause of death in non-coronary intensive-care units (Thiemermann, 1997). Since AS is the rate-limiting enzyme in both the urea (Morris, 1992) and the arginine–citrulline (Xie & Gross, 1997) cycles, it is a prime target for inhibiting NO production. The observation that inhibitors of NO production can reverse hypotension in animal models (Kilbourn, Gross *et al.*, 1990; Kilbourn, Jubran *et al.*, 1990) suggests that inhibitors of AS could be of therapeutic value in the treatment of septic and cytokine-induced shock.

Escherichia coli AS is active as a homotetramer of approximately 50 kDa subunits and has 28.8% sequence identity and 44.9% similarity to the human enzyme using the Blosum 50 (Henikoff & Henikoff, 1992) scoring matrix. From the crystal structures of GMP (Tesmer *et al.*, 1996) and NAD$^+$ (Rizzi *et*

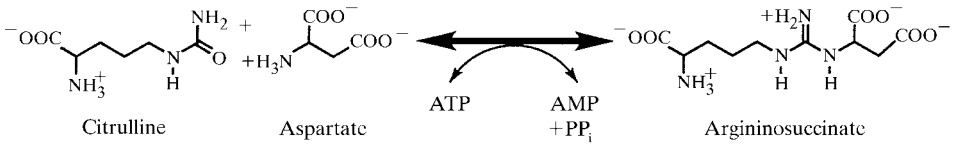

Figure 1
The reaction catalyzed by argininosuccinate synthetase.

al., 1996) synthetase, it was recognized that AS was a member of a new class of enzymes which adenylate their substrates to promote subsequent amination reactions. These 'N-type' ATP pyrophosphatases share a glycine-rich consensus-sequence motif that forms a modified P-loop of a nucleotide-binding domain similar to that of GTP/ATP-dependent proteins. While classical P-loops interact with the single terminal phosphate of the nucleotide, this new P-loop has been shown to bind the pyrophosphate moiety and was hence dubbed the PP-motif (Bork & Koonin, 1994; Rizzi *et al.*, 1996; Tesmer *et al.*, 1996). The structure of AS will not only enable the function of the newly described PP-motif to be verified, but will also provide the fold of the synthetase domain and a model from which the enzymatic mechanism of this protein can be determined. At present, little is known about the synthetase domain, as it has no sequence similarity to any protein of known structure. The crystallization and preliminary X-ray analysis of recombinant *E. coli* argininosuccinate synthetase reported here represent the first essential steps towards determining the structure of this enzyme.

2. Expression and purification

The *E. coli* argininosuccinate synthetase (EAS) gene was the gift of Dr F. Van Vliet (Vrije University, Brussels, Belgium). The EAS coding sequence was amplified by PCR such that *Nde*I and *Sal*I restriction sites were introduced at the N- and C-termini, respectively. The PCR product was ligated into the pET21b expression vector (Novagen) at the compatible *Nde*I and *Xho*I restriction sites such that a 6-histidine tag linked by the tripeptide Ser-Val-Glu was appended to the enzyme. DNA sequencing was performed on the coding region of the resulting construct to ensure that the correct nucleic acid sequence had been conserved through the PCR and subsequent genetic manipulations. The resulting expression vector, pET21b-EAS, was transformed into *E. coli* strain BB101 {genotype *ara* Δ(*lac proAB*) Δ*slyD* (*kan*^{r}) *nalA argEam rif thi* F′[*lacI*^{q} *proAB*$^+$] (λDE3)}. The *slyD* deletion of this strain prevents the expression of a 21 kDa histidine-rich *E. coli* protein which binds strongly to the nickel affinity resin used to purify the EAS protein. Starter cultures were prepared by inoculating 5 ml of LB medium containing 100 μg ml^{-1} ampicillin and incubating overnight at 310 K. Each overnight growth was then pelleted and resuspended in 1 ml fresh medium and then diluted in 1 l of LB medium containing 100 μg ml^{-1} ampicillin. This culture was then grown at 310 K to an OD_{600} of 0.3–0.4, at which point protein expression was induced by the addition of IPTG to a final concentration of 1 m*M*. The cells were harvested 3–4 h post-induction by centrifugation (7000 rev min^{-1}, 277 K, 15 min) and then resuspended in 50 ml buffer *A* (500 m*M* NaCl, 20 *M* Tris–HCl pH 7.9). The cells were then lysed by ten rounds of sonication comprised of 30 s pulses followed by 60 s of cooling on ice. The soluble fraction of the lysate was decanted after further centrifugation (28000*g*, 277 K, 25 min) and loaded directly onto a 3 ml His-Bind Ni^{2+} affinity column (Novagen) previously equilibrated with buffer *A*. The column was first washed with 30 ml buffer *A* with 5 m*M* imidazole, then with 20 ml buffer *A* containing 40 m*M* imidazole. The protein was eluted from the column with buffer *A* containing 200 m*M* imidazole in a single 15 ml fraction. The buffer was changed to 25 m*M* Tris–HCl pH 7.5 and the protein was concentrated to approximately 20 mg ml^{-1} using an Ultrafree-15 Biomax-10K NMWL membrane concentrator (Millipore). Protein purity was assessed using SDS–PAGE (Fig. 2) and a single band of the expected molecular weight, 50 kDa, was observed. Although a substantial amount of protein was found in the pellet, this protocol yielded approximately ∼20 mg of 99% pure soluble enzymatically active protein from 1 l of bacterial culture.

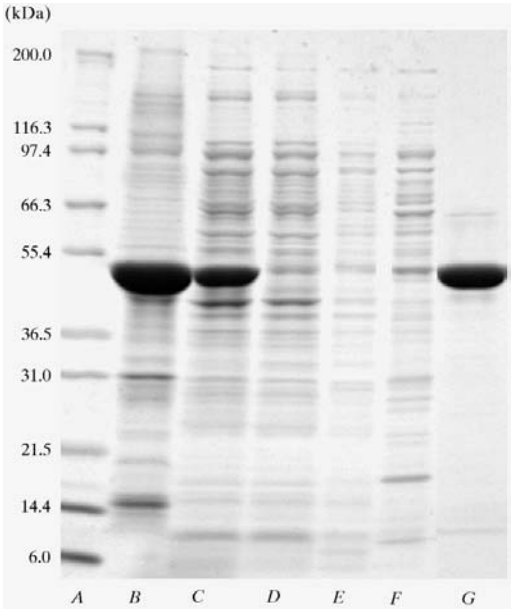

Figure 2
Coomassie-stained SDS–PAGE gel showing EAS purification. Lane *A*, molecular-weight markers; lane *B*, insoluble lysate; lane *C*, soluble lysate; lane *D*, flow-through; lane *E*, 5 m*M* imidazole wash; lane *F*, 40 m*M* imidazole wash; lane *G*, 200 m*M* imidazole wash.

3. Crystallization

All crystals were grown using the hanging-drop vapour-diffusion technique at room temperature (293 K). The initial screening for crystallization conditions was performed using the sparse-matrix screens 1 and 2 from Hampton Research (Jancarik & Kim, 1991). Small crystals were obtained from several varied conditions, a number of which led to diffraction-quality crystals upon optimization. The simplest of these conditions produced good-quality crystals that diffracted to high resolution. To produce these crystals, equal volumes of protein solution (10 mg ml^{-1}, 25 m*M* Tris–HCl pH 7.5) and precipitating solution (1.5–1.6 *M* ammonium sulfate, 100 m*M* MES pH 6.5) were mixed on a siliconized glass cover slip and suspended over a 1 ml reservoir containing the same precipitating solution. Crystals appear within 7–14 d and grow to approximately 1.0 × 0.5 × 0.5 mm (Fig. 3).

4. X-ray analysis

Prior to data collection, a crystal (0.4 × 0.3 × 0.2 mm) was soaked in a 30%(*v*/*v*) glycerol solution, 1.6 *M* ammonium sulfate, 100 m*M* MES pH 6.8 for approximately 3 min then transferred to a rayon CryoLoop (Hampton Research) and flash-frozen in liquid nitrogen. The frozen crystal was subsequently mounted in a continuous cold stream and data were collected at 100 K on a MAR345 image plate at station X8C at the National Synchrotron Light Source, Brookhaven National Laboratory, NY, USA. Two sets of data were collected, the first to maximize the signal obtained at high reso-

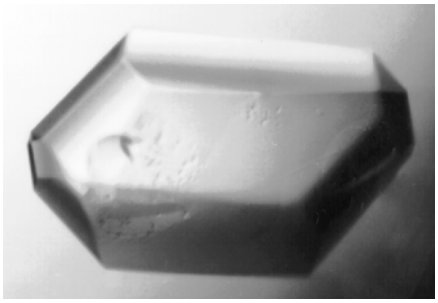

Figure 3
Crystal of *E. coli* argininosuccinate synthetase. The crystal is approximately 0.9 × 0.5 × 0.3 mm.

Table 1
Diffraction data statistics.

Values given in parentheses refer to reflections in the outer resolution shell, 1.66–1.60 Å.

No. of measured reflections	688919
No. of independent reflections	70186
Resolution (Å)	1.6
$R_{\rm merge}$†	4.2 (20.7)
Completeness (%)	98.7 (87.4)
Completeness [$>3I/\sigma(I)$] (%)	90.7 (65.6)
Average $I/\sigma(I)$	34.7

† Defined as $R = \sum |I_k - \langle I \rangle| / \sum I(k)$, where $I(k)$ and $\langle I \rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean values. The summation is over all measurements.

lution and the second to ensure that the low-resolution intensities were accurately measured. For the low-resolution data, a total of 120 images of 2° $\Delta\varphi$ oscillations were collected (*i.e.* 240° of data). For the high-resolution data, 179 and 73 images of 1.25° and 1.5° $\Delta\varphi$ oscillations were recorded, respectively. Preliminary autoindexing and refinement of the cell and setting parameters were performed using the program *DENZO* (Otwinowski & Minor, 1997). The unit-cell parameters were found to be a = 79.70, b = 105.84, c = 127.33 Å, $\alpha = \beta = \gamma = 90°$. The full data-reduction statistics are presented in Table 1. Examination of the systematic absences uniquely determined the space group to be $I222$. On the basis of density calculations (V_m = 2.67 Å^3 Da^{-1}; Matthews, 1968), we estimate that one monomer of the tetrameric protein is present in the asymmetric unit. We are currently in the process of determining the structure of this protein using the multi-wavelength anomalous diffraction technique (Hendrickson, 1991).

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