

Purification, crystallization and preliminary X-ray analysis of aspartokinase III from *Escherichia coli*

Julio Blanco and Ronald E. Viola*

Department of Chemistry, University of Toledo,
2801 West Bancroft Street, Toledo, Ohio 43606,
USA

Correspondence e-mail: ron.viola@utoledo.edu

Aspartokinase III catalyzes the commitment step in the aspartate metabolism pathway, the phosphorylation of aspartic acid. The *Escherichia coli* enzyme has been crystallized in the presence of its natural substrate (aspartic acid) and Mg-ADP and diffraction data has been collected at a synchrotron source. The crystals belong to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 60.44$, $b = 190.31$, $c = 99.55$ Å, and data 99.3% complete to 2.7 Å. Solving the structure of AK III will provide the first structure of an aspartokinase from any organism.

Received 30 May 2001

Accepted 3 December 2001

1. Introduction

The aspartate pathway uses aspartic acid as the precursor for the biosynthesis of the amino acids lysine, methionine, isoleucine and threonine (Cohen, 1983). Although absent in mammals, this pathway is essential in plants and microorganisms. In addition to these essential amino acids there are several important metabolic intermediates derived from this pathway, including diaminopimelic acid, a key component required for cross-linking in bacterial cell-wall biosynthesis, and dipicolinic acid, important for sporulation in Gram-positive bacteria. Organisms utilizing this pathway often contain more than one aspartokinase that catalyzes the initial step in the pathway, the phosphorylation of L-aspartic acid. These isofunctional aspartokinases are subject to differential regulation, both by feedback inhibition and by repression at the genetic level, from the end-product amino acids. In many of these organisms at least one of these enzymes is bifunctional, catalyzing both the first reaction, the phosphorylation of aspartate and, surprisingly, the third reaction, the reduction of aspartate semialdehyde to homoserine, in this metabolic sequence. In *E. coli* there are three aspartokinases, two of which are bifunctional enzymes; the other, aspartokinase III (AK III), is a monofunctional enzyme inhibited by high lysine levels (Cohen & Dautry-Varsat, 1980). The sequence of the AK III catalytic domain has 44% homology to the bifunctional aspartokinase domains. The homology between the N-terminal domains of the three aspartokinases suggests a common ancestor for the genes encoding these proteins.

We have been examining the central enzymes of the aspartate pathway (Viola, 2001) to determine their catalytic and regula-

tory mechanisms. The aspartokinases have fairly broad substrate specificity and can catalyze the phosphorylation of a range of aspartate analogs, including an unusual reversal of regioselectivity for β -derivatized analogs (Angeles *et al.*, 1992). Chemical modification and pH-profile studies (Keng & Viola, 1996) have suggested several catalytically important functional groups; however, mutagenesis studies have not yet succeeded in identifying the essential catalytic residues. An earlier report on the crystallization of the bifunctional aspartokinase I has been published (Janin, 1974), but no subsequent progress on the structure has been reported since then.

2. Experimental

2.1. *E. coli* AK III expression, purification and characterization

The AK III gene (*lysC*; 1350 bases) from *E. coli* was introduced into the pTZ19U expression vector using *Hind*III and *Eco*RI restriction endonucleases, which create a linkage at the initiator site and downstream of the stop codon, respectively. The construct was verified by DNA sequencing to carry the complete *lysC* gene and was subsequently transformed into BL21(DE3) competent cells (Novagen). The cells were grown in TB broth in the presence of ampicillin ($50 \mu\text{g ml}^{-1}$) at 310 K until A_{600} reached about 0.6. The culture was then induced with 1 mM IPTG and agitated at 250 rev min^{-1} for an additional 4 h. The cells were harvested by centrifugation at 9000g for 30 min and the subsequent purification steps were carried out at 277 K.

The cell pellet was resuspended in buffer A (10 mM potassium phosphate pH 6.8, 100 mM KCl, 1 mM L-lysine, 2 mM MgCl_2 , 1 mM L-threonine, 1 mM EDTA, 0.1 mM DTT) and

disrupted by sonification. The crude extract was clarified by centrifugation (9000g, 20 min) and brought to 20% saturation by the incremental addition of solid ammonium sulfate. An active supernatant was separated by centrifugation (9000g, 20 min) then brought to 40% saturated ammonium sulfate as described above. The protein pellet was solubilized in 50 ml buffer *A* and then dialyzed to remove the ammonium sulfate. The protein solution containing the AK III activity was loaded onto a 50 ml HiTrap Q-Sepharose XL (Pharmacia Biotech) column equilibrated with buffer *A* using an Äkta Explorer 100 chromatography system. Aspartokinase III was eluted at approximately 300 mM KCl in a linear gradient of buffer *A* containing 0.9 M KCl. The partially purified AK III sample was dialyzed against buffer *B* (50 mM HEPES pH 7.0, 1 mM EDTA, 1 mM DTT, 1 M ammonium sulfate) and applied to a 75 ml ω -aminohexyl agarose (Sigma) column equilibrated with buffer *B*. Pure AK III was eluted from this hydrophobic resin at approximately 0.5 M ammonium sulfate in a linear gradient with buffer *B* minus the ammonium sulfate. Enzyme purity (subunit MW = 50 kDa) was assessed by densitometer scanning (UN-SCAN-IT, Silk Scientific Inc.) of the SDS-PAGE. The enzyme was concentrated by ultrafiltration (Millipore) to 15 mg ml⁻¹ and dialyzed in 50 mM HEPES pH 7.0, 25 mM dipotassium hydrogen phosphate, 5 mM DTT, 1 mM EDTA, 5 mM ADP, 30 mM L-aspartic acid, 5 mM magnesium acetate for storage or subsequent crystallization trials.

AK III activity was measured by coupling the formation of β -aspartyl phosphate with aspartate semialdehyde dehydrogenase (ASA DH). The reaction mixture consisted of 120 mM HEPES buffer pH 8.0, 200 mM KCl, 5 mM magnesium acetate, 4.0 mM

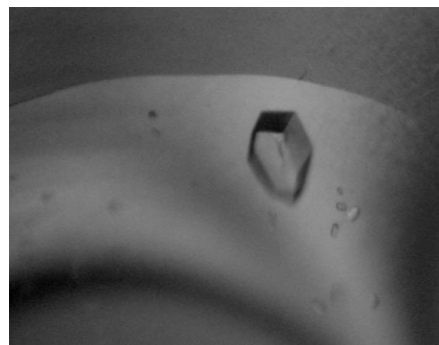


Figure 1
Crystals of AK III. Typical crystals grew to dimensions of $0.6 \times 0.2 \times 0.2$ mm and diffracted to a maximum resolution of 2.7 Å at the synchrotron source.

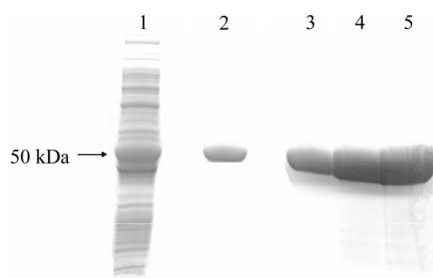


Figure 2
Expression and purification of AK III. SDS-PAGE of lane 1, cell extracts of *E. coli* BL21(DE3) cells transformed with pTZ19UlysC; lane 2, 5 µg of protein after anion exchange; lanes 3, 4 and 5, increasing amounts of purified enzyme (10, 15 and 20 µg, respectively).

ATP, 0.1 mM NADPH, 15 mM L-aspartic acid, 0.5–1 µg AK III and 20–30 µg ASA DH. Initial velocities were measured by monitoring the oxidation of NADPH at 340 nm.

2.2. Crystallization and X-ray data collection

Initial crystallization conditions were tested by the sitting-drop vapor-diffusion method using Crystal Screen kits (Hampton Research) in multiwell plates by mixing 5 µl reservoir fluid and 5 µl of 15 mg ml⁻¹ AK III. The best crystals (Fig. 1), reaching at least 0.5 mm in the largest dimension, were produced in about 10 d at 298 K with 18% PEG 3350, 0.2 M ammonium nitrate and 0.1 M Tris pH 8.5. Harvesting solutions were 14% PEG 3350, 0.2 M ammonium nitrate, 5 mM ADP, 10 mM magnesium acetate, 30 mM L-aspartic acid and 20% glycerol in 0.1 M sodium citrate pH 5.6.

Preliminary diffraction of these crystals was examined on a R-AXIS IV and a full data set was collected on an MAR Research 345 imaging plate at Argonne National Laboratory BioCARS beamline (14-D). The distance of the image plate was set to 175 mm and the images were recorded with 1° oscillation per image and an exposure time of 15 s per frame.

3. Results and discussion

Purification of AK III, as described in §2, leads to about 75 mg of enzyme which is greater than 99% pure as judged by densitometer scanning of the SDS-PAGE (Fig. 2). The crystals

Table 1
Data-collection parameters.

Values in parentheses are for the highest resolution shell.	
X-ray source	APS BioCARS beamline 14-D
Wavelength (Å)	1.0
Temperature (K)	90
Space group	<i>C222</i> ₁
Unit-cell parameters (Å)	<i>a</i> = 60.44, <i>b</i> = 190.31, <i>c</i> = 99.55
Resolution range (Å)	2.7 (2.8–2.7)
Total No. of reflections	67703
No. of unique reflections	16035
Redundancy	4.22
Completeness	99.3 (99.9)
<i>R</i> _{merge} (%)	7.8 (33.2)
<i>I</i> / σ (<i>I</i>)	12.76 (3.08)
Volume of asymmetric unit (Å ³)	1139813
No. of monomers per asymmetric unit	1

of AK III that were obtained were found to be somewhat sensitive to radiation damage. These crystals were tested against a wide range of potential cryoprotectant solutions to minimize radiation damage by allowing data collection at low temperatures. The addition of 20% glycerol was found to stabilize the crystals, with minimal deterioration during freezing.

The diffraction images (Fig. 3) were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystals belong to the orthorhombic space group *C222*₁, with unit-cell parameters *a* = 60.44, *b* = 190.31, *c* = 99.55 Å (Table 1). One AK III monomer (50 kDa) per asymmetric unit yields a Matthews coefficient of 2.8 Å³ Da⁻¹ (Matthews, 1968) and an approximate solvent content of 56%. A complete native



Figure 3
The diffraction pattern of AK III. Data were collected at APS BioCARS beamline 14-D at a detector distance of 175 mm and a 15 s exposure. The resolution at the edge of the figure is approximately 2.0 Å.

data set has been obtained to 2.7 Å, corresponding to an R_{merge} of 7.8%. If the molecule is organized as a dimer, then the molecular twofold symmetry axis should be coincident with the crystallographic dyad axis. Details of the data-collection statistics are summarized in Table 1.

Both heavy-atom derivatives (for MIR phasing) and the incorporation of anomalous scattering atoms (for MAD phasing) are being examined. Solving the structure of AK III will provide the first structure of an aspartokinase from any organism. Armed with this initial structure as a probe and the recently reported structure of the homo-

serine dehydrogenase from yeast, which is 35% identical to *E. coli* HDH (DeLaBarre *et al.*, 2000), the structure of the more complex bifunctional aspartokinase-homoserine dehydrogenases will be pursued.

The authors thank Drs Gloria Borgstahl, Kabaleeswaran Venkataraman and Tim Mueser (University of Toledo) for helpful discussions.

References

- Angeles, T. S., Hunsley, J. R. & Viola, R. E. (1992). *Biochemistry*, **31**, 799–805.
- Cohen, G. N. (1983). *Amino Acids: Biosynthesis and Genetic Regulation*, edited by K. M. Herrmann & R. L. Somerville, pp. 147–171. Reading, MA, USA: Addison-Wesley.
- Cohen, G. N. & Dautry-Varsat, A. (1980). *Multifunctional Proteins*, edited by H. Bisswanger & E. Schmincke-Ott, pp. 49–121. New York: John Wiley & Sons.
- DeLaBarre, B., Thompson, P. R., Wright, G. D. & Berghuis, A. M. (2000). *Nature Struct. Biol.* **7**, 238–244.
- Janin, J. (1974). *FEBS Lett.* **45**, 318–319.
- Keng, J. F. & Viola, R. E. (1996). *Arch. Biochem. Biophys.* **335**, 73–81.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Viola, R. E. (2001). *Acc. Chem. Res.* **34**, 339–349.