Purification, Crystallization, and Preliminary X-ray Analysis of Aspartokinase III from *Escherichia coli*

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Introduction

The aspartate pathway uses aspartic acid as the precursor for the biosynthesis of the amino acids lysine, methionine, isoleucine, and threonine [1]. Although absent in mammals, this pathway is essential in plants and microorganisms. In addition to these essential amino acids, several important metabolic intermediates are 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 using 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 and, surprisingly, the third reaction in this metabolic sequence. *Escherichia coli* has three aspartokinases, two of which are bifunctional enzymes. The other one, aspartokinase III (AK III), is a monofunctional enzyme inhibited by high lysine levels [2].

We have been examining the central enzymes of the aspartate pathway [3] to determine their catalytic and regulatory 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 [4]. Chemical modification and pH profile studies [5] 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 was published [6], but no subsequent progress on the structure has been reported since then.

Methods and Materials

AK III Expression and Purification

The AK III gene (lysC, 1350 bases) from *E. coli* was introduced into the pTZ19U expression vector by using *Hin*d III and *Eco*R I restriction endonucleases. *Hin*d III creates a linkage at the initiator site, and *Eco*R I creates a linkage downstream of the stop codon. 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 µL/mL) at 310K until A_{600} reached about 0.6. The culture was then induced with 1 mM IPTG and agitated at 250 rpm for an additional 4 h. The cells were harvested by centrifugation at 9000 g for 30 min, and the subsequent purification steps were carried out at 277K. 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 at 9000 g for 20 min and then brought to 20% saturation by the incremental addition of solid ammonium sulfate. An active supernatant was separated by centrifugation at 9000 g for 20 min and then brought to 40% saturated ammonium sulfate as described above. The protein pellet was solubilized in 50 mL of 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 by 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 molecular weight [MW] = 50 kilodalton [kDa]) was assessed by densitometer scanning (UN-SCAN-IT, Silk Scientific, Inc.) of the SDS-PAGE (Fig. 1). 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.

Enzyme Assay

AK III activity was measured by coupling the formation of β-aspartyl phosphate with aspartate

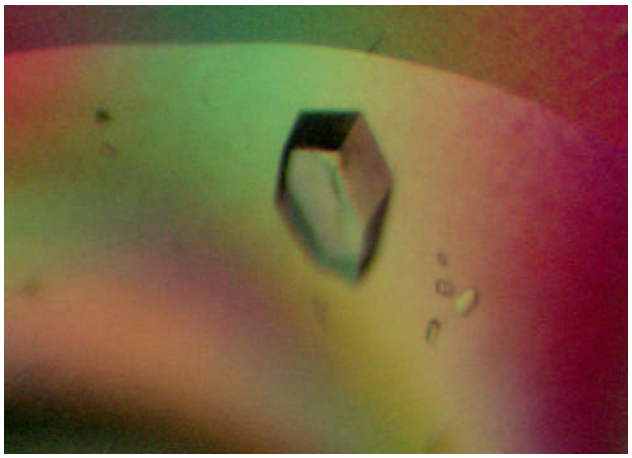

FIG. 1. Crystals of AK III. Typical crystals were grown up to 0.6 ´ 0.2 ´ 0.2 mm, and they diffract to a maximum resolution of 2.7 Å at the synchrotron source.

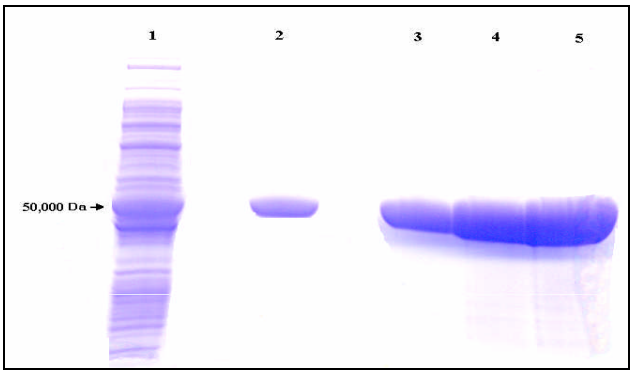

FIG. 2. Expression and purification of AK III. SDS-PAGE of the following: Lane 1 = extracts of E. coli BL21(DE3) cells transformed with pTZ19UlysC, Lane 2 = 5 µg of protein after anion exchange, and Lanes 3-5 = increasing amounts (10, 15, 20 µg) of purified enzyme.

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 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.

Crystallization and X-ray Data Collection

Initial crystallization conditions were tested by the sitting-drop vapor-diffusion method by 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 298K 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 at pH 5.6.

Preliminary diffraction of these crystals was examined on a Raxis IV, and a full data set was collected on a MAR Research 345 imaging plate at beamline 14-D of the Bio Consortium for Advanced Radiation Sources (BioCARS) at the APS. 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.

Results and Discussion

Purification of AK III, as described in the section discussing the experiments, leads to about 75 mg of enzyme that is more than 99% pure, as judged by densitometer scanning of the SDS-PAGE (Fig. 2). The crystals 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 liquid nitrogen 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 by using the programs DENZO and SCALEPACK [7]. The crystals belong to the orthorhombic space group $C222_1$, with unit cell parameters a = 60.44 Å, b = 190.31 Å, and c = 99.55 Å (Table 1). One AK III monomer (50 kDa) per asymmetric unit yields to a Matthews coefficient of 2.8 $Å^3$ [8] and an approximate solvent content of 56%. A complete native data set has been obtained to 2.7 Å, corresponding to an R_{merge} of 7.8%. Details of the data collection statistics are summarized in Table 1.

Both heavy atom derivatives (for multiple isomorphous replacement [MIR] phasing) and the incorporation of anomalous scattering atoms (for multiwavelength

Table 1. Data collection parameters.

Data set	Native enzyme
X-ray source	BioCARS beamline 14-D
Wavelength (Å)	1.0
Temperature (K)	293
Space group	$C222_1$
Unit-cell parameters (Å)	a = 60.44, b = 190.31, c = 99.55
Resolution range (Å)	2.7 [2.8-2.7]
Completeness	99.3 [99.9]
R_{merge} (%)	7.8 [33.2]
No. of unique reflections	16,035
Vol. of asym. unit ($Å^3$)	1,139,813
Monomers per asym. unit	1

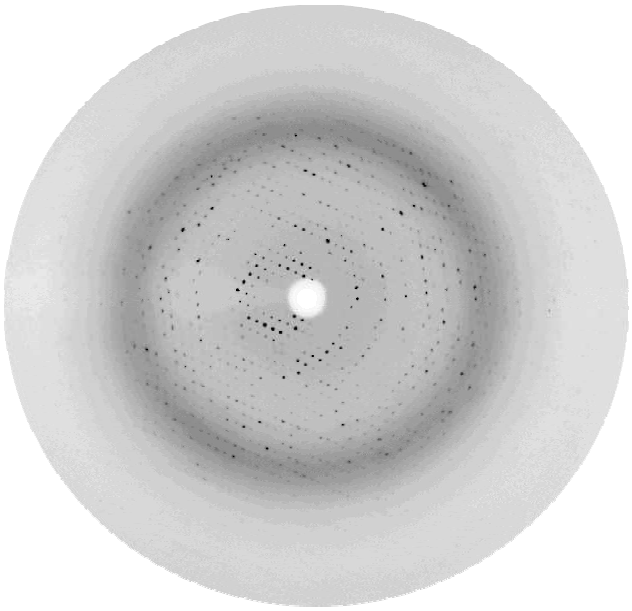

FIG. 3. The diffraction pattern of AK III. Data were collected at BioCARS beamline 14-D at a detector distance of 175 mm and a 15-s exposure. The resolution at the edge of the figure is 2.0 Å.

anomalous dispersion [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 homoserine dehydrogenase from yeast [9], the structure of the more complex bifunctional aspartokinase-homoserine dehydrogenases will be pursued.

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