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FOR THE RECORD

Crystallization and preliminary structural analysis of *Bacillus subtilis* adenylosuccinate lyase, an enzyme implicated in infantile autism

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Abstract: Adenylosuccinate lyase (ASL) from *Bacillus subtilis* has been crystallized and structural analysis by X-ray diffraction is in progress. ASL is a 200-kDa homotetramer that catalyzes two distinct steps of de novo purine biosynthesis leading to the formation of AMP and IMP; both steps involve the β-elimination of fumarate. A single point mutation in the human ASL gene has been linked to mental retardation with autistic features. In addition, ASL plays an important role in the bioprocessing of anti-HIV therapeutics. *B. subtilis* ASL, which shares 30% sequence identity and 70% sequence similarity with human ASL, has been crystallized and data to 3.3 Å have been collected at 100 K. The space group is $P6_122$ or $P6_522$ with $a = b = 129.4$ Å; the length of the c-axis varies between 275 and 290 Å, depending on the crystal. An analysis of solvent content indicates a dimer in the asymmetric unit, although a self-rotation function and an analysis of native Pattersons failed to identify unambiguously the location of any noncrystallographic symmetry axes. Structure determination by isomorphous replacement is in progress.

Keywords: autism; fumarase; HIV therapeutics; purine biosynthesis; X-ray crystallography

Adenylosuccinate lyase (ASL; E.C. 4.3.2.2; also known as adenylosuccinase) catalyzes two steps of the de novo biosynthesis of purine ribonucleotides, both involving the removal of fumarate (Seegmiller, 1980). In one reaction, ASL catalyzes the conversion of 5-amino-4-imidazole-*N*-succinylocarboxamide ribotide (SAICAR) to 5-amino-4-imidazolecarboxamide (AICAR). This is the ninth step in biosynthesis of IMP from ribose 5′-phosphate (Fig. 1A). In the second reaction, ASL catalyzes the conversion of adenylosuccinate to AMP (Fig. 1B). Several disease states have been associated with defects in the purine biosynthetic pathway, including Down's syndrome and sensorineuronal deafness (Patterson et al., 1981; Becker et al., 1986). ASL defects have been associated with mental retardation with autistic features (Jaeken & Van den Berghe, 1984; Jaeken et al., 1988). In ASL-deficient patients, succinyladenosine and SAICA riboside (degradative products of ASL substrates adenylosuccinate and SAICAR) accumulate in body fluids, whereas unaffected subjects show no detectable levels (<1 μM) of such compounds. Stone et al. (1992) identified a single point mutation (T to C) in the human ASL gene that leads to a Ser-413 to Pro (S413P) alteration in the enzyme. All three of the mentally retarded children in a Moroccan family were homozygous for this mutation. The parents and unaffected children in the family were heterozygous and the alteration was absent in control subjects. The S413P mutation leads to decreased enzyme stability, as evidenced by its greater heat lability and its increased sensitivity to guanadinium HCl and urea (Stone et al., 1992). Neither the binding of adenylosuccinate and SAICAR to ASL nor the kinetic parameters of the enzyme were affected by this mutation. Decreased levels of ASL are detected (by western analysis) in the lymphocytes of affected patients relative to unaffected individuals (Stone et al., 1992). It has been proposed that the mutant enzyme is more prone to degradation by cellular proteases than its native counterpart (Stone et al., 1992).

ASL also plays an important role in the conversion of dideoxypurine nucleosides to dideoxynucleotide triphosphates with known anti-HIV (human immunodeficiency virus) activ-

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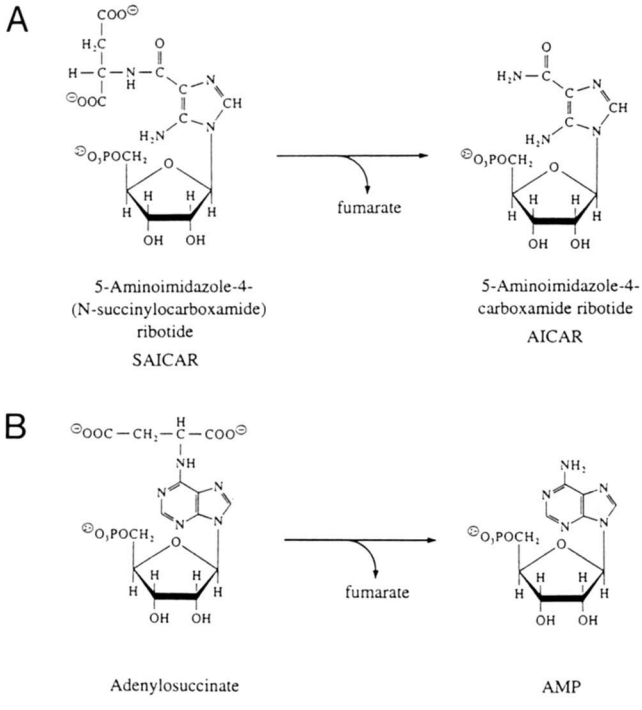

Fig. 1. The two reactions in de novo purine biosynthesis catalyzed by adenylosuccinate lyase (ASL). **A:** Ninth step in the 11-step de novo biosynthesis of IMP from ribose-5-phosphate. **B:** Second step in the conversion of IMP to AMP.

ity (Nair & Sells, 1992). 2′,3′-dideoxyadenine (ddA) and 2′,3′-dideoxyinosine (ddI) have shown promise in vitro as therapeutic agents that slow the proliferation of the virus (Mitsuya & Broder, 1986). ddI has undergone phase II screening and has been approved for clinical trials (Mitsuya et al., 1990; Moyle et al., 1993). During activation of ddI within the cell, ASL has been found to convert ddSAMP with only 2% efficiency relative to its physiological substrate, adenylosuccinate (Nair & Sells, 1992).

The ASL gene has been isolated from the bacteria *Escherichia coli* and *Bacillus subtilis*, and cDNAs have been isolated from avian (chicken) liver and human liver (Ebbole & Zalkin, 1987; Aimi et al., 1990; Stone et al., 1992). The human and avian enzymes are both 459 amino acids, 52.3 kDa, and are active as tetramers. The *Bacillus* enzyme is 431 amino acids, displays a relative monomer molecular weight of 52 kDa, and is also active as a tetramer. The human and avian enzymes share 85% sequence identity, whereas the human and *Bacillus* enzymes are 30% identical. The catalytic mechanisms of these enzymes are similar. There are several significant stretches of sequence identity present in all three enzymes, including an 11-amino acid putative "signature sequence" (263-GSSAMP[Y/H]KRND-274 for ASL) found in enzymes that catalyze β-eliminations of fumarate (Stone et al., 1993). A putative superfamily of metabolic enzymes has been identified that includes δ-crystallines, fumarases, aspartases, argininosuccinate lyase, adenylosuccinate lyase, and 3-carboxy-*cis*,*cis*-muconate lactonising enzyme. These enzymes are all 200-kDa homotetramers and carry out β-elimination reactions (Simpson et al., 1994). The crystal structures of turkey δ-1 crystallin and *E. coli* fumarase C have been determined to 2.5 Å and 2.0 Å resolution, respectively, and reveal homotetrameric enzymes with 222 symmetry (Simpson et al., 1994; Weaver et al., 1995). δ-1 crystalline and fumarase C may represent structural models for this superfamily of metabolic enzymes.

The gene encoding *B. subtilis* ASL was engineered with PCR to encode six amino-terminal histidyl residues and placed under the transcriptional control of the φ10 promoter of the pT7-7 expression vector (Tabor & Richardson, 1985). The plasmid was introduced into *E. coli* strain BL21 (DE3) for protein production. Briefly, a 1-L culture of transformed bacteria was grown at 37 °C in rich medium and 100 μg/mL ampicillin to an absorbance reading at 600 nm of 0.5. IPTG was added to a final concentration of 500 μM and incubation was continued for an additional 3 h. Bacteria were harvested by centrifugation at 20,000 × *g* for 20 min. Recombinant *B. subtilis* ASL was purified to homogeneity from the crude lysate in a single step on NTA-agarose saturated with Ni^{2+}. Elution was accomplished with a 0–500 mM imidazole gradient in 50 mM Tris-HCl, pH 6.0, 300 mM NaCl, following extensive washing with the same buffer lacking imidazole. Chromatographic fractions containing enzymatic activity were pooled and concentrated to 15 mg/mL in 20 mM sodium phosphate buffer, pH 7.0, 20 mM NaCl.

Crystals were obtained at 22 °C in 1.3–1.5 M ammonium sulfate, 0.1 M MES buffer at pHs 5.5, 5.6, and 5.8–6.2. Crystallization trials at pH 5.7 yielded only amorphous precipitate. The crystals are strongly birefringent hexagonal bipyramids, and grow to a maximal size of 1.2 × 0.8 × 0.8 mm in 1–3 weeks from a standard hanging-drop vapor diffusion experiment (Fig. 2). A ratio of 2:1 for protein to well solution was found to produce the largest crystals, and crystals of similar morphology and quality were found to grow in the presence or absence of 1 mM AMP added to the protein solution.

The crystals diffract X-rays to approximately 4.5 Å resolution at 22 °C on an R-AXIS IIc imaging plate with a Rigaku rotating anode source (100 kV, 50 mA, Cu-Kα radiation, 1.54-Å wavelength), but decay rapidly in the beam. Cryoprotection of the crystals in 35–40% glycerol and the mother liquor used to

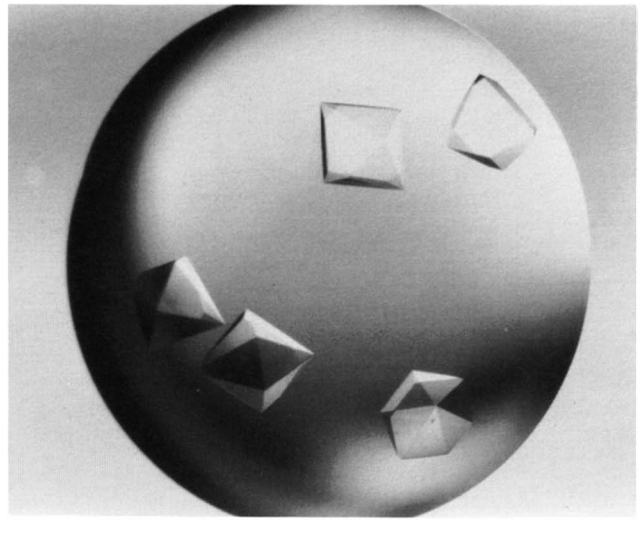

Fig. 2. Hexagonal bipyramidal crystals of *B. subtilis* ASL. These crystals grew in 1.5 M ammonium sulfate, 0.1 M MES buffer, pH 5.8, in two weeks at 22 °C, and measure approximately 0.8 × 0.6 × 0.6 mm.

support crystal growth allows cooling of the crystals to 100 K in nitrogen gas. This extends the diffraction resolution to approximately 3.8 Å resolution on an in-house R-AXIS imaging plate and effectively eliminates the problem of crystal decay. The unit cell was determined to be hexagonal, with $a = b = 129.4$ Å, $c \approx 280$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$, by automated indexing with R-AXIS software and with the program DENZO (Otwinowski & Minor, 1995). The length of the *c*-axis varies from 275 to 290 Å depending on the crystal. A native data set has been collected to 3.3 Å resolution at 100 K using 1.15-Å wavelength radiation at beamline X12C, National Synchrotron Light Source, Brookhaven National Laboratory (*c*-axis = 282.1 Å, R_{merge}[6] = 6.1%, 93% complete, $\langle I/\sigma \rangle = 5.4$ for data between 3.4 and 3.3 Å resolution). The space group was determined to be either $P6_122$ or $P6_522$ by examination of systematic absences, and an analysis of the Matthew's parameter ($V_M = 3.4$ Å^3/Da) indicated a dimer in the asymmetric unit (Matthews, 1968). However, a self-rotation function (Navaza, 1994) and an analysis of native Pattersons (both using data to 3.3 Å resolution) failed to identify the location of noncrystallographic symmetry axes.

B. subtilis ASL does not share a high degree of sequence identity with any proteins of known structure. *B. subtilis* ASL is 22% identical over 431 amino acids to the chicken δ-I crystallin sequence, which was built into the crystal structure of turkey δ-I crystallin (chicken and turkey δ-I crystallin share approximately 90% sequence identity) (Simpson et al., 1994). *E. coli* glutathione synthetase, for which a 2.0-Å crystal structure has been reported (1GLT; Yamaguchi et al., 1993), is 18% identical to *B. subtilis* ASL over a 122-amino acid stretch. As expected, this 122-amino acid fragment produced no useful molecular replacement solutions using the automated molecular replacement package AMoRe (Navaza, 1994). Structure determination of *B. subtilis* ASL is progressing using the isomorphous replacement method.

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$$^{6}R_{merge} = \frac{\sum_{r}^{N}\left[\sum_{i}^{n(r)} |I_{i,r} - \langle I_r \rangle|\right]}{\sum_{r}^{N} n(r)\langle I_r \rangle},$$

where N is the number of unique reflections, $I_{i,r}$ is the ith measurement of the rth reflection, $\langle I_r \rangle$ is the average of the rth reflection, and $n(r)$ is the number of measurements of the rth reflection of the data set.

References

Aimi J, Badylak J, Williams J, Chen Z, Zalkin H, Dixon JE. 1990. Cloning of a cDNA encoding adenylosuccinate lyase by functional complementation in *Escherichia coli*. *J Biol Chem 265*:9011–9014.

Becker MA, Losman MJ, Rosenberg AL, Mehlman I, Levinson DJ, Holmes EW. 1986. Phosphoribosylpyrophosphate synthetase superactivity. A study of five patients with catalytic defects in the enzyme. *Arthritis Rheum 29*:880–888.

Ebbole DJ, Zalkin H. 1987. Cloning and characterization of a 12-gene cluster from *Bacillus subtilis* encoding nine enzymes for de novo purine nucleotide synthesis. *J Biol Chem 262*:8274–8287.

Jaeken J, Van den Berghe G. 1984. An infantile autistic syndrome characterized by the presence of succinylpurines in body fluids. *Lancet ii*: 1058–1061.

Jaeken J, Wadman SK, Duran M, van Sprang FJ, Beemer FA, Holl RA, Theunissen PM, de Cock P, Van den Berghe F, Van den Berghe G. 1988. Adenylosuccinase deficiency: An inborn error of purine nucleotide synthesis. *Eur J Pediatr 148*:126–131.

Matthews BW. 1968. Solvent content of protein crystals. *J Mol Biol 33*:491–497.

Mitsuya H, Broder S. 1986. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. *Proc Natl Acad Sci USA 83*:1911–1915.

Mitsuya H, Yarchoan R, Broder S. 1990. Molecular targets for AIDS therapy. *Science 249*:1533–1544.

Moyle GJ, Nelson MR, Hawkins D, Gazzard BG. 1993. The use and toxicity of didanosine (ddI) in HIV antibody-positive individuals intolerant to zidovudine (AZT). *Quart J Medicine 86*:155–163.

Nair V, Sells TB. 1992. Interpretation of the roles of adenylosuccinate lyase and of AMP deaminase in the anti-HIV activity of 2′,3′-dideoxyadenine and 2′,3′-dideoxyinosine. *Biochim Biophys Acta 1119*:201–204.

Navaza J. 1994. AMoRe: An automated package for molecular replacement. *Acta Crystallogr A 50*:157–163.

Otwinowski Z, Minor W. 1995. *The HKL manual*. New Haven, Connecticut: Yale University.

Patterson D, Graw S, Jones C. 1981. Demonstration, by somatic cell genetics, of coordinate regulation of genes for two enzymes of purine synthesis assigned to human chromosome 21. *Proc Natl Acad Sci USA 78*:405–409.

Seegmiller JE. 1980. Diseases of purine and pyrimidine metabolism. In: Bondy PK, Rosenberg LE, eds. *Metabolic control and disease*. Philadelphia, Pennsylvania: WB Saunders. pp 777–937.

Simpson A, Bateman O, Driessen H, Lindley P, Moss A, Mylvaganam S, Narebor E, Slingsby C. 1994. The structure of avian eye lens δ-crystallin reveals a new fold for a superfamily of oligomeric enzymes. *Nature Struct Biol 1*:724–734.

Stone RL, Aimi J, Barshop BA, Jaeken J, Van den Berghe G, Zalkin H, Dixon JE. 1992. A mutation in adenylosuccinate lyase associated with mental retardation and autistic features. *Nature Genetics 1*:59–63.

Stone RL, Zalkin H, Dixon JE. 1993. Expression, purification, and kinetic characterization of recombinant human adenylosuccinate lyase. *J Biol Chem 268*:19710–19716.

Tabor S, Richardson CC. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA 82*:1074–1078.

Weaver TM, Levitt DG, Donnelly MI, Wilkens Stevens PP, Banaszak LJ. 1995. The multisubunit active site of fumarase C from *Escherichia coli*. *Nature Struct Biol 2*:654–662.

Yamaguchi H, Kato H, Hata Y, Nishioka T, Kimura A, Oda J, Katsube Y. 1993. Three-dimensional structure of the glutathione synthetase from *Escherichia coli* at 2.0 Å resolution. *J Mol Biol 229*:1083–1100.