Two Novel Mutant Human Adenylosuccinate Lyases (ASLs) Associated with Autism and Characterization of the Equivalent Mutant Bacillus subtilis ASL*

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An Australian patient with autism was found to be heterozygous for two mutations in the gene encoding adenylosuccinate lyase (ASL), resulting in the protein mutations E80D and D87E. The patient’s mother carried only the E80D mutation. The equivalent positions are 62 and 69 in Bacillus subtilis ASL. Although both human and B. subtilis enzymes normally have Asp at position 87 (or 69), the B. subtilis ASL has Ile and Asp at 62 and 65, respectively, whereas human ASL has Glu and Arg at the equivalent positions. We have constructed, expressed, and purified the double mutant I62E/D65R as a “humanized” normal B. subtilis enzyme to compare with enzymes with a single mutation at position 62 (I62D/D65R), at position 69 (I62E/D65R/D69E), or at both positions (I62D/D65R/D69E). Vmax for conversion of adenylosuccinate to AMP and fumarate is 0.57 μmol/min/mg for I62E/D65R, 0.06 μmol/min/mg for I62D/D65R, 0.27 μmol/ min/mg for I62E/D65R/D69E, and 0.069 μmol/min/mg for I62D/D65R/D69E. The Ks for adenylosuccinate is elevated in the X62D mutants, and I62D/D65R is the least stable of these ASLs at 37 °C. The CD spectra of mutant and wild type enzymes are similar; thus, there are no appreciable structural changes. Clearly the AspE62 causes the most drastic effect on ASL function, whereas the GluD69 mutation produces only modest change. These results emphasize the importance of expanding tests for ASL deficiency to individuals with developmental delay of any severity, including individuals with autistic spectrum disorder. This study further demonstrates the usefulness of the B. subtilis ASL as a model to mimic the defective enzyme in ASL deficiency.

Adenylosuccinate lyase deficiency is an inborn error of metabolism resulting from a defective enzyme important in the de novo pathway of purine biosynthesis (1, 2). This deficiency is characterized by variable degrees of developmental delay, often accompanied by autistic features and epileptic seizures (2). Biochemically, the enzyme deficiency is diagnosed by the appearance in cerebrospinal fluid, urine, and to a smaller extent in plasma, of succinylaminoimidazole carboxamide riboside (SAICA-riboside)† and succinyladenosine (S-Ado)‡. ASL catalyzes two distinct reactions in the synthesis of purine nucleotides, as shown in Scheme Ia, the cleavage of adenylosuccinate (SAMP) to AMP, the second step in the conversion of IMP to AMP and, Scheme Ib, the conversion of succinylaminoimidazole carboxamide ribotide (SAICAR) to aminoimidazole carboxamide ribotide (AICAR), the eighth step of the de novo synthesis (3). The two reactions proceed by β-elimination of fumarate via a general base-general acid mechanism, where the general base abstracts a methylene proton from the carbon in the β-position relative to the leaving nitrogen, and the general acid donates a proton to the leaving nitrogen. The succinyladenosine and SAICA-riboside detected in the ASL-deficient patients are the dephosphorylated derivatives of S-Ado and SAICAR, respectively. In severely affected patients, the concentrations of the compounds are comparable, whereas in more mildly affected patients, the concentration of SAICA-riboside is in the same range, but the succinyladenosine concentration is much higher, resulting in S-Ado/SAICA riboside ratios of greater than 2 (2, 4). To date, ~60 patients with ASL deficiency have been diagnosed worldwide, but there may be many more because there has not been general screening for this disease, especially in cases of mild developmental delay or autism.

This study focuses on a recently identified individual from Australia with ASL deficiency, diagnosed with autism at the age of 2; it was not until the age of 6 years that he experienced 2 epileptic seizures and was subsequently treated with an anticonvulsant (5). The patient has elevated levels of S-Ado and SAICA-riboside in the urine (normally undetectable), consistent with mild ASL deficiency. We report here the identification of the mutations in the ASL of this patient. To gain insight into the effect of these mutations on the catalytic and structural features of the enzyme, we have introduced these mutations into the ASL from Bacillus subtilis at the positions equivalent to those of the human enzyme.

The human gene encoding ASL is located on chromosome 22q13.2. To date, more than 30 different mutations in the ASL gene causing this deficiency have been identified worldwide. The mutations resulting in ASL deficiency are located throughout the enzyme, with the majority of them being relatively far from the active site. However, biochemical study of the human ASL has been conducted in only a limited number of cases (7–9) because of the instability of the enzyme. As an alternative, because of the high structural similarity between the enzymes...
in this family, we have previously used the more stable B. subtilis ASL as a model system and have constructed in the bacterial enzyme other mutations corresponding to those associated with human ASL deficiency (10, 11). ASL is a homotetramer with single chains containing 431–487 amino acids, depending on the source, with a molecular mass of ~200 kDa. The active site of the ASL of B. subtilis has been examined by affinity labeling and site-directed mutagenesis, implicating in the active site His88 and His141 (12–14), His89 (15), Lys268 and Glu275 (16), and Gln212, Asn270, and Arg201 (17). The ASL of Thermotoga maritima has been crystallized, and its coordinates are deposited in the Protein Data Bank (18). The B. subtilis and T. maritima enzymes share 50% identity plus 23% similarity in amino acid sequence, whereas the B. subtilis and human ASL share 25% identity plus 23% similarity in sequence. Homology models of the B. subtilis (16) and human enzymes (11) have been constructed based on the crystal structure of T. maritima ASL. The structural models of these three enzymes are superimposable (11) and have been used to relate four human enzyme amino acid residues previously identified in ASL-deficient patients to B. subtilis amino acid residues (10, 11). We report here two new disease-associated mutations and the characterization of mutant enzymes with substitutions at positions 62 and 69 of the B. subtilis ASL to provide insight into the effects of the corresponding new disease-associated mutations found in the human enzymes sequenced in this paper. A preliminary version of this work has been presented (19).

EXPERIMENTAL PROCEDURES

Materials—SAMP, AICAR, HEPES, and imidazole were purchased from Sigma. Oligonucleotides for both sequencing and site-directed mutagenesis were obtained from Biosynthesis. The protein assay concentration was from Bio-Rad. All other chemicals were of reagent grade.

Identification of Adenylosuccinate Lyase Deficiency Mutations—PCR-amplified DNAs corresponding to each exon from the affected individual and mother were sequenced. The DNA of the father was not available. Genomic DNA was isolated from whole peripheral blood cells (20).

The exons of ASL were amplified using the PCR primers described in Kmoch et al. (8), with slight modifications. The PCR products were either purified using a Roche Applied Science High Pure Purification kit and sequenced directly or cloned using the Promega pGEM-T Easy Vector and Novagen NovaBlue competent cells and purified using the Promega Wizard Plus Miniprep and sequenced using an ABI Big Dye Terminator Cycle Sequencing kit. Sequences were read on either an ABI Prism 377 DNA sequencer or an ABI 373 Stretch DNA sequencer.

Site-directed Mutagenesis—Mutations to the pBHis plasmid (a gift from Dr. Jack E. Dixon, University of Michigan) that encodes adenylosuccinate lyase of B. subtilis were constructed using the Stratagene QuickChange mutagenesis kit. The following oligonucleotides and their complements were used to generate the Ile62 mutant enzymes: CGC ATT TTA GAA GAA GAA AAG GAC ACG CG (I62E) and C CGC ATT TTA GAA GAT GAA AAG GAC ACG CG (I62D). The Asp65 mutant enzyme was generated from the following oligonucleotide and its complement: C ACG CGC CAT GAA ATC GAA GAT GAA AAG GAC ACG CG (Asp/Arg), respectively. The I62E/D56R and I62D/D56R mutant enzymes were constructed using the corresponding Ile62 mutant cDNA as the template and the following oligonucleotides and their complements: GAA GAA GAA AAG GAC ACG CG (I62E), GAA GAT GAA AAG GAC ACG CG (I62D), and GAA GAT GAA AAG GAC ACG CG (I62E/D56R, I62D/D56R, and D65R/D69E mutant enzymes were constructed using the corresponding Ile62 mutant cDNA as the template and the following oligonucleotides and their complements: GAA GAA GAA AAG GAC ACG CG (I62E), GAA GAT GAA AAG GAC ACG CG (I62D), and GAA GAT GAA AAG GAC ACG CG (I62E/D56R, I62D/D56R, and D65R/D69E mutant enzymes were constructed using the D69E oligonucleotide and its complement on the I62E/D65R and I62D/D65R mutant cDNA templates, respectively. The I62E/D65R, I62D/D65R, and D65R/D69E mutant enzymes were constructed using the D69E oligonucleotide and its complement on the I62E, I62D, and D65R mutant cDNA templates, respectively. The mutations were confirmed by DNA sequencing, carried out at the Delaware Biotechnology Institute and University of Delaware Center for Agricultural Biotechnology using an ABI Prism model 377 DNA sequencer (PE Biosystems).

The pBHis plasmid, which encodes a His6 tag on the N-terminus of adenylosuccinate lyase, was expressed in Escherichia coli strain BL21 (DE3), and the enzymes were purified to homogeneity using Qiagen nickel nitriotriatic acid-agarose (12, 21). The purity of the enzymes was evaluated by 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, and the protein concentrations were determined by the absorbance at 280 nm using $E_{280}^{\text{nm}} = 10.6$ (12). The purified enzyme was separated into aliquots and stored at −80 °C in 20 mM potassium phosphate containing 20 mM sodium chloride, pH 7.
SAICAR Synthesis—SAICAR was prepared enzymatically from AICAR (10 mM) and fumarate (100 mM) with adenylosuccinate lyase (0.4 mg/ml) in 50 mM HEPES, pH 7.0, reaction mixture (2.5 ml total volume). The reaction was allowed to proceed for 2 h and was monitored on the UV-visible spectrophotometer (Hewlett Packard 8453) by the decrease in absorbance of AICAR at 267 nm and increase in absorbance of SAICAR at 269 nm. The enzyme was removed by filtering the reaction mixture using a Centricron-10 filtration device (Millipore). The filtrate was then applied to a DEAE-cellulose column (1.5 × 43 cm) equilibrated with 10 mM NH₄HCO₃, pH 7.9, and the compounds were eluted using a linear gradient from 10 mM (1 liter) to 300 mM (1 liter) NH₄HCO₃, pH 7.9. Of the three peaks seen, the first and second peaks are attributed to the unreacted reagents fumarate and AICAR, respectively. The product SAICAR elutes as the third peak, detected by the absorbance at 269 nm. The fractions from the third peak were pooled, evaporated to dryness under vacuum, redissolved in water, and applied to a Dowex-50 column (24 × 1 cm) (Bio-Rad A 50W-X4, 100–200 mesh, hydrogen form) equilibrated with 0.1 M HCl to desalt the product.

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Circular Dichroism Spectroscopy—The Jasco J-710 spectropolarimeter was used to measure ellipticity as a function of wavelength from 250 to 200 nm in 0.2-nm increments using a 0.1-cm cylindrical quartz cuvette. The wild type and mutant enzymes were incubated at 0.4 mg/ml for 30 min at 25 °C before measuring the spectra, and the final protein concentration was determined by the Bradford protein assay, which is based on the method of Bradford, using wild type adenylosuccinate lyase as the protein standard (24). The samples were scanned five times and averaged, and the spectrum of the buffer, containing 20 mM potassium phosphate and 20 mM sodium chloride at pH 7, was subtracted. The mean molar ellipticity [θ] (deg cm² dmol⁻¹) was calculated from the equation [θ] = θ/10 nCl, where θ is the measured ellipticity in millidegrees, C is the molar concentration of enzyme subunits, l is the path length in centimeters, and n is the number of residues per subunit (457, including the His6 tag).

Thermal Stability of Wild Type and Mutant Enzymes—The wild type and mutant enzymes were incubated at 1 mg/ml at 37 °C, and 20–200 μl aliquots were assayed (depending on the activity of the enzyme) under standard conditions every 10 min for the first hour and every 15 min for the next 3 h. All the enzyme samples were preincubated for 30 min at 25 °C in 20 mM potassium phosphate buffer, pH 7.0, containing 20 mM sodium chloride before being transferred to 37 °C.

Molecular Weight Determination by Light Scattering—The wild type and mutant enzyme molecular weights were determined using a mIDAWN laser photometer (Wyatt Technology Corp., Santa Barbara, CA). The molecular weights were determined at a protein concentration of 0.25 mg/ml in 20 mM potassium phosphate containing 20 mM sodium chloride, pH 7. The protein concentrations were determined by Bio-Rad protein assays after collecting data. The data were collected at room temperature at the laser wavelength of 690 nm and analyzed using ASTRA software for Windows (25).

RESULTS

Identification of Point Mutations in Adenylosuccinate Lyase-deficient Patient—To determine the mutations present in the ASL gene of the affected child, PCR amplified genomic DNA corresponding to each exon from the affected individual and mother was sequenced. The DNA sequences of the relevant region from the mother and proband are shown in Fig. 1. Two mutations were found in the proband, and one in the parent; both are on exon 2 of the ASL gene. The first mutation is an A

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FIG. 1. DNA sequence from mother and proband in the relevant region. The trinucleotide regions leading to the amino acid substitutions E80D and D87E are boxed, and the mutant nucleotides are marked by red arrows.
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The mutant enzymes were expressed in E. coli and purified to homogeneity. Representative enzyme samples are shown on the SDS-PAGE gel in Fig. 3.

Kinetics of Mutant Enzymes—All the mutant B. subtilis enzymes constructed exhibit sufficient activity to be characterized kinetically. The \( V_{\text{max}} \) and \( K_m \) values using SAMP as substrate are summarized in Table I. The \( V_{\text{max}} \) value of the 162E mutant enzyme (which was expected to be close to that of the normal enzyme) is very low when compared with that of the wild type B. subtilis ASL (lines 1 and 2). In the B. subtilis enzyme model, the decrease in activity could be due to the repulsion between the negatively charged group at position 87, whereas the Glu at position 87 is not conserved.

Accordingly, all disease-associated mutations are best compared with the enzyme of line 9 (I62E/D65R). The most informative mutant enzymes are those shown in lines 9–12: line 10 (as compared with line 9), line 11 (as compared with line 9), I62E/D65R and I62D/D65R, exhibit appreciably higher \( V_{\text{max}} \) values. The original Asp\textsuperscript{65} residue of the B. subtilis enzyme apparently does have an unfavorable interaction with the negatively charged amino acid at position 62 in the 162E and 162D enzymes.

The mutant enzyme of line 9 (with 62E, 65R and 69D) is the best representation of a normal humanized B. subtilis ASL. Accordingly, all disease-associated mutations are best compared with the enzyme of line 9 (I62E/D65R). The most informative mutant enzymes are those shown in lines 9–12: line 10 (as compared with line 9), line 11 (as compared with line 9), I62E/D65R and I62D/D65R, exhibit appreciably higher \( V_{\text{max}} \) values.

The \( K_m \) value for glutamate (GAA) to aspartate (GAC) substitution at amino acid 80 (E80D). Both the affected individual and the parent are heterozygous for this mutation. The second mutation is a T to G substitution at nucleotide 242 resulting in an aspartate (GAT) to glutamate (GAG) substitution at amino acid position 87 (D87E). The mother has the wild type coding sequence for D87, whereas the proband is heterozygous for the mutation encoding D87E. No other nucleotide changes were found in the ASL genes of the patient or the mother.

Equivalence Mutations in B. subtilis to the Adenylosuccinate Lyase Deficiency-associated Mutations Found in Humans—This study focuses on two point mutations identified in the child with adenylosuccinate lyase deficiency (5). As shown in Fig. 2, amino acid residues 80 and 87 in the human sequence correspond, respectively, to amino acids 62 and 69 of the B. subtilis enzyme based on sequence alignment generated using ClustalW. A complete sequence alignment of the adenylosuccinate lyases from H. sapiens, B. subtilis and T. maritima was published previously (11). The two point mutations in the human enzyme are E80D and D87E, and the corresponding residues in the B. subtilis enzyme are Ile\textsuperscript{62} and Asp\textsuperscript{69}, thus, the Asp at position 87 in the normal human enzyme is conserved, but the Glu at position 80 is not conserved. Therefore, 162E was constructed to mimic the normal human enzyme at positions 80 and 87. I62D was generated to correspond to the single mutation of the ASL-deficient child at position 80, and D69E was designed to correspond to the single mutation of the affected child at position 87.

In addition, we noted that B. subtilis adenylosuccinate lyase has a negatively charged aspartate at position 65, whereas the equivalent amino acid in the human enzyme is the positively charged arginine and the corresponding amino acids in the T. maritima, mouse, and chicken, according to CLUSTALW.

FIG. 2. Amino acid sequence alignments of adenylosuccinate lyase from B. subtilis, human, T. maritima, mouse, and chicken, according to CLUSTALW.

FIG. 3. SDS-PAGE of representative purified enzymes. The contents of each lane are as follows: lane 1, protein standards; lane 2, WT; lane 3, I62E; lane 4, I62D; lane 5, D69E; lane 6, I62E/D65R; lane 7, I62D/D65R; lane 8, I62E/D65R/D69E; lane 9, I62D/D65R/D69E; and lane 10, I62D/D69E. Approximately 5 µg of each sample was applied to the gel.

TABLE I. Kinetic values for adenylosuccinate (line 4) versus D69E (line 5) mutant enzymes. The mixture gave a \( V_{\text{max}} \) value of 0.12 mol substrate/min/mg protein (data not shown), which is almost twice that of the double mutant (line 12). Table I also reports the \( k_{\text{cat}} \) values for wild type and the various mutants.

Compared with the wild type \( K_m \) of 4 µM for adenylosuccinate, the humanized normal mutant enzyme (line 9), I62E/D65R, has a 4-fold higher \( K_m \) whereas the 162D change results in a 14-fold higher \( K_m \) when present as a single mutation (line 3). This higher \( K_m \) value is also seen in the humanized 162D/D69E single mutant (line 11). The double defect mutant has a \( K_m \) lower than that of a single I62D mutant (line 12 versus line 11; line 6 versus line 3). The effect of the single mutation of D69E is to reduce the \( K_m \) for adenylosuccinate (line 4 versus line 1; line 10 versus line 9). Thus it is not surprising that the \( K_m \) of the double mutant reflects the favorable contribution of D69E to the apparent affinity for substrate. The D65R mutant enzyme has a \( K_m \) value that is similar to that of the wild type enzyme (line 7 versus 1). Compared with the catalytic efficiency...
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The activities were measured from the decrease in absorbance at 282 nm using SAMP in 50 mM HEPES, pH 7, at 25 °C. The $K_m$ values were calculated by varying the SAMP concentration from 5 to 150 $\mu$M, and the $V_{\text{max}}$ was determined by extrapolation to saturating concentrations of substrate.

| Enzyme | $V_{\text{max}}$ | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ |
|--------|-----------------|-----------------|------|-----------------|
|        | $\mu$mol min$^{-1}$ mg$^{-1}$ | s$^{-1}$ | $\mu$M | $\mu$M s$^{-1}$ |
| 1. Wild type | 1.87 ± 0.04 | 1.57 ± 0.03 | 4.0 ± 0.7 | 3.90 × 10$^5$ |
| 2. I62E | 0.05 ± 0.003 | 0.07 ± 0.003 | 17.5 ± 2.8 | 0.03 × 10$^5$ |
| 3. I62D | 0.03 ± 0.01 | 0.03 ± 0.008 | 55.1 ± 10 | 0.005 × 10$^5$ |
| 4. D69E | 0.41 ± 0.009 | 0.34 ± 0.008 | 1.3 ± 0.6 | 2.64 × 10$^5$ |
| 5. I62E/D69E | 0.10 ± 0.02 | 0.08 ± 0.002 | 4.3 ± 0.8 | 0.19 × 10$^5$ |
| 6. I62D/D69E | 0.03 ± 0.01 | 0.03 ± 0.001 | 17.7 ± 2.2 | 0.01 × 10$^5$ |
| 7. D65R | 1.27 ± 0.07 | 1.07 ± 0.006 | 4.6 ± 1.4 | 2.31 × 10$^5$ |
| 8. D65R/D69E | 0.24 ± 0.01 | 0.20 ± 0.008 | 2.0 ± 0.4 | 1.00 × 10$^5$ |
| 9. I62E/D65R | 0.57 ± 0.004 | 0.48 ± 0.003 | 18.1 ± 2.4 | 0.26 × 10$^5$ |
| 10. I62E/D65R/D69E | 0.27 ± 0.01 | 0.23 ± 0.008 | 6.1 ± 1.3 | 0.37 × 10$^5$ |
| 11. I62D/D65R | 0.06 ± 0.002 | 0.05 ± 0.002 | 48.8 ± 9.7 | 0.01 × 10$^5$ |
| 12. I62D/D65R/D69E | 0.07 ± 0.005 | 0.06 ± 0.004 | 10.7 ± 2.4 | 0.05 × 10$^5$ |

* Humanized normal B. subtilis enzyme.
* 69 mutant (mimics human defect at 87).
* 62 mutant (mimics human defect at 80).
* Double defect.

$(k_{\text{cat}}/K_m)$ of the normal human-like enzyme, the I62D/D65R mutant enzyme is 25-fold less efficient (line 9 versus 11) whereas the I62E/D65R/D69E mutant enzyme exhibits a minimal change in $k_{\text{cat}}/K_m$ (line 9 versus 10). Therefore, the $V_{\text{max}}$ and $K_m$ values of the ASL-deficient mutant enzymes are predominantly affected by the I62D single point mutation.

**SAICAR/SAMP Ratios of Wild Type and ASL Deficiency-associated Mutant Enzymes**—In some patients with ASL deficiency, there is a differential effect on the enzyme’s catalytic activity toward SAMP and SAICAR (2). The relative activities toward the 2 substrates are compared in Table II for the mutants constructed in the present study. The activities of the wild type and all mutant enzymes are similar for the two substrates tested. The activities of the humanized single defect mutant at 69 (I62E/D65R/D69E), double defect mutant (I62D/D65R/D69E) and the heterozygous enzyme mixture (I62D/D65R + I62E/D65R/D69E) exhibit only a slightly higher SAICAR/SAMP ratio.

**Circular Dichroism Spectroscopy of Wild Type and Mutant Enzymes**—Circular dichroism was used to detect any major changes in the secondary structure of the mutant enzymes that might be responsible for the altered catalytic activities. The CD spectra of all the mutant B. subtilis enzymes are shown in Fig. 4. The wild-type and the single point mutations equivalent to those identified in the affected child are shown in Fig. 4A. The humanized enzymes with ASL deficiency single defect at 62, 69, and the double defect (equivalent to lines 9–12, Table I) are shown in Fig. 4B. The control mutant enzymes for all the ASL deficiency mutations are shown in Fig. 4C. Wild type and all mutants exhibit minima in ellipticity at 208 and 222 nm, typical of proteins containing $\alpha$-helices. Overall, there is no appreciable difference in the CD spectra of the mutant enzymes.

**Molecular Weight Determination of Wild Type and Mutant Enzymes**—To evaluate the effect of the disease-associated mutations on the oligomeric state of the enzyme, light scattering was used. Because the molecular weights depend on protein concentration (10), the determinations were made at the same concentration (−0.25 mg/ml), yielding the molecular weights shown in Table III. The results show that the molecular weights are consistent with an equilibrium mixture between the dimeric and tetrameric structure of the wild type and mutant enzymes, as we have previously demonstrated for wild type enzyme (10).

Light scattering could not be used on the humanized enzymes with single mutations at position 62, I62E/D65R and I62D/D65R, because of their tendency to aggregate. Therefore, the molecular weights of these enzymes were determined using native gel electrophoresis (11), based on the relative mobility ($R_M$) of the samples in the 5−9% polyacrylamide gels. The calculated molecular mass of the wild type enzyme is 210 kDa. The I62E/D65R and I62D/D65R mutant enzymes exhibit distinct bands of 209 kDa and 217 kDa, respectively, as the smallest species present; however, they also exhibit an appreciable amount of higher molecular weight bands of about 381 kDa and 336 kDa, respectively. These results are consistent with the thermal instability of these mutant enzymes.

**Thermal Stability Studies of Wild Type and ASL Deficiency-associated Human Mutant Enzymes**—To evaluate the effect of the ASL deficiency-associated point mutations on the stability of the enzyme, the rates of inactivation of wild type and mutant enzymes were tested at 37 °C, chosen since it is close to the physiological temperature. The time-dependent inactivations are illustrated in Fig. 5 and the rate constants are summarized in Table IV. Of the ASL deficiency-associated point mutations studied, I62D/D65R is the most unstable enzyme, with a rate constant for inactivation of 0.025 min$^{-1}$. Neither the wild type nor the normal humanized enzyme (I62E/D65R) is completely stable over the time period studied. The ASL deficiency double mutant (I62D/D65R/D69E) enzyme is the second most unstable mutant with $k = 0.012$ min$^{-1}$, compared with the wild type and normal humanized enzymes of 0.003 min$^{-1}$ and 0.009 min$^{-1}$, respectively. Therefore, the I62D mutation seems to have the most detrimental effect on the stability of the enzyme.

**DISCUSSION**

The present study identifies two new point mutations associated with adenylosuccinate lyase deficiency found in an affected individual in Australia and characterizes them using the B. subtilis enzyme as a model. The physical features of the mutant enzymes did not show an appreciable difference from wild type enzyme in the secondary and overall enzyme structure. But there are significant differences in thermal stability, catalytic activity and substrate binding in these mutant enzymes. When compared with the normal human-like enzyme (I62E/D65R), the single mutant at position 69 (I62E/D65R/D69E) causes only a 2-fold decrease in $V_{\text{max}}$ whereas a single defect at 62 (I62D/D65R) results in a 9-fold reduction in $V_{\text{max}}$. The double defect (I62D/D65R/D69E) mutant is very similar in $V_{\text{max}}$ to the X62D mutant. An equal mixture of the two single defect mutant enzymes, which is the better representation of
the affected individual, has a 2-fold higher $V_{\text{max}}$ than the double defect enzyme. Also, the lowered $V_{\text{max}}$ values of I62E and I62D mutant enzymes suggest that Asp65 in the *B. subtilis* causes an unfavorable interaction with the negatively charged residues at 62 in I62E and I62D. Therefore, it was important to change Asp to Arg at position 65 to mimic the human enzyme. The normal human-like enzyme has a 4-fold higher $K_m$ than wild type *B. subtilis*, but the X62D mutation causes about a 14-fold increase in $K_m$. The catalytic efficiency of the human like X62D mutant enzyme is 25-fold less than the normal human like enzyme. Therefore, the mutation at position 62, which corresponds to human mutation E80D, is more deleterious than the mutation at position 69, which corresponds to human mutation D87E.

### TABLE II

*Comparison of adenylosuccinate and SAICAR activities of wild type and mutant enzymes*

The enzyme activity for the two substrates were each measured using 90 μM substrate in 50 mM HEPES, pH 7, at 25 °C.

| Enzyme                        | SAMP     | SAICAR   | SAICAR/SAMP |
|-------------------------------|----------|----------|-------------|
| Wild type                     | 1.58     | 1.70     | 1.07        |
| I62E/D65R                    | 0.54     | 0.53     | 0.97        |
| I62E/D65R/D69E               | 0.33     | 0.46     | 1.40        |
| I62D/D65R                    | 0.063    | 0.069    | 1.09        |
| I62D/D65R/D69E               | 0.072    | 0.094    | 1.30        |
| I62D/D65R + I62E/D65R/D69E   | 0.18     | 0.24     | 1.29        |

**FIG. 4.** Circular dichroism spectra of wild type and mutant enzymes. These spectra were determined with each protein (∼0.25 mg/ml) in 20 mM potassium phosphate buffer, pH 7, containing 20 mM sodium chloride. A, single-point mutations. B, human-like mutations. (C) Control mutations.
Based on the crystal structure of the *T. maritima* enzyme, homology models of the mutant enzymes were constructed in the *B. subtilis* enzyme. The energy minimized models of the wild type and mutant enzymes are superimposable, indicating no major conformational changes have taken place in the backbone structure of the enzymes. But there are some notable local changes, which are shown in Fig. 6. The normal human-like enzyme model is shown in Fig. 6A. The mutant enzymes with a single defect at position 62 or 69 are shown in Fig. 6B and Fig. 6C, respectively. Previous studies have shown protonated His\(^{68}\) to be a critical active site residue (14), which promotes the elimination reaction by acting as a general acid and also facilitates SAMP binding by interacting with the two carboxylates of its succinyl moiety. We propose that Asp\(^{69}\) must interact with His\(^{68}\) to raise its pK and keep it in its protonated state. Mutations at 62 and 69 indirectly change the distances between the active site residue His\(^{68}\) and the -COO\(^{-}\) and -COO\(^{-}\) of SAMP, as well as between Asp\(^{69}\) and His\(^{68}\). This distortion of the enzyme-substrate complex may cause the low activity and binding seen in these mutant enzymes. In the I\(^{62D}/D^{65R}\) model (Fig. 6B), Lys\(^{260}\) from the adjoining subunit is only 3.03 Å from X\(^{62D}\) compared with 5.55 Å in the normal human-like enzyme with an Glu at position 62 (Fig. 6A). This intersubunit perturbation indirectly increases the distance between Asp\(^{69}\) and His\(^{68}\) and changes the relative distances between His\(^{68}\) and the two succinyl carboxylates. This perturbation of the subunit interactions may cause the instability of this mutant enzyme. In the I\(^{62E}/D^{65R}/D^{69E}\) model (Fig. 6C), the 69 to 68 distance is closer to that of the normal human-like enzyme, but the relative proximity of the His\(^{68}\) and the two succinyl carboxylates is still altered (one...
distance is greater and the other smaller). This distortion of an active site residue, His^656, and perturbation in the subunit interactions at Lys^626 may be responsible for the ASL deficiency disease seen in this patient.

ASL deficiency has been classified into two types: Type I exhibits severe disease, whereas Type II has milder clinical features. In both the levels of S-Ado and SAICA-riboside are elevated, but in Type I the ratio of S-Ado/SAICA-riboside is ~1 and in Type II the ratio is 2 or greater (2). The affected individual has a S-Ado/SAICA-riboside ratio of 2.4, a value consistent with mild ASL deficiency. Studies on other ASL deficiency mutants have shown that in some cases the relative activity of the enzyme toward the two substrates is related to the severity of the disease and to the ratio of the dephosphorylated compounds found (9). However, the results shown here suggest that this classification system may be an oversimplification: the mutant enzymes mimicking those of the proband, have lost activity equally for both substrates. This result is consistent with the observations reported by Kmoch et al. (8) in which the mutant enzymes displayed proportional decrease in activity toward both substrates, yet the individual patients had different concentration ratios of the dephosphorylated forms of the two substrates in the cerebrospinal fluid. It may be that a mild clinical presentation can be accompanied by parallel loss of enzyme activity. The distinction between Type I and Type II disease may be more complex than simply the differential loss of activities with the two substrates. The various ratios of the dephosphorylated compounds found in biological fluids could be due to a secondary effect, such as unequal rates of dephosphorylation or unequal rates of transport of the dephosphorylated compounds.

So far, all identified cases of ASL deficiency have an autosomal recessive pattern of inheritance. There have been no abnormalities reported for any of the parents of ASL patients, and the mother of the patient described here appears to be clinically unaffected. One patient has been reported who carries a 39 base pair deletion of the ASL gene leading to deletion of amino acids 206–218 in the human sequence (27). The deletion enzyme was reported to be inactive (9). Therefore, it appears that 50% of normal ASL activity, as would be present in the parent of this individual that carries the deletion allele, is sufficient for normal health and development. An observation consistent with this interpretation was made by Marie et al., where a mutation in the ASL promoter of a normal ASL allele coupled with a missense mutation in the other allele led to severe ASL deficiency in three ASL deficiency patients (28). The promoter mutation was found to result in about 33% of normal transcription of ASL gene. In this case, the parent of each patient that carried the promoter mutation on one allele and one normal allele would have 67% of normal ASL activity. In the present case, the mother of the patient carries the E80D mutant allele. The equivalent B. subtilis mutant, I62D/D65R, has about 12% of normal human-like enzyme activity with either SAMP or SAICAR. The mother, with the equivalent of one X62D mutant allele, would be expected to possess well over 50% of normal ASL activity. This is consistent with the observation that the mother is clinically unaffected.

The affected individual is heterozygous for the two mutations. Assuming that there is equal expression and stability of the two mutant enzymes in the proband (unlikely given the decreased stability of the mutant enzymes), the residual activity in the proband would be ~30%. This is similar to the residual ASL activity found in many ASL patients (8, 9, 26).

Recent studies on understanding disease-causing mutations in humans have shown that disease mutations are more likely to occur at amino acid residues that are highly conserved throughout evolution (29, 30). In interspecific substitutions, a Glu to Asp and Asp to Glu change is found to be quite common, and rarely associated with human genetic diseases. The mutations studied here are not completely consistent with this observation. The B. subtilis residue Asp^656, which corresponds to human mutation D87E, is completely conserved in different species, whereas Ile^626, which corresponds to human mutation E80D, is not. Even though the normal amino acid found at position 80 is not highly conserved, Asp is never found at this position. One could speculate that this is because a Asp at this position would inactivate the enzyme and thus be selected against during evolution. This would be consistent with the finding that the E80D mutation leads to severe loss of enzyme activity.

Recently, an individual with a novel, neurologically devastating inborn error of purine biosynthesis caused by a mutation in AICAR transformylase/IMP cyclohydrolase (ATIC) was described by Marie et al. (31). This individual was initially diagnosed by the Bratton-Marshall test (32), the most commonly used diagnostic assay for ASL deficiency, which led to the identification of massive excretion of AICA-riboside, the dephosphorylated counterpart of AICAR, a product of ASL and substrate for ATIC. AICAR transformylase activity is undetectable in this individual, who survived at least until 4 years of age. Our results and the finding of a mutation in another step of de novo purine synthesis leading to neurological disease strongly reinforce the notion that testing for ASL deficiency (and deficiencies in other steps of de novo purine synthesis) should be expanded to individuals with developmental delay of any severity, including individuals with autistic spectrum disorder. The Bratton-Marshall test would provide a convenient initial screen, but would need to be accompanied by additional methods such as HPLC (2, 6), which is also used in ASL deficiency, because mutations in steps of the pathway that could be due to a secondary effect, such as unequal rates of dephosphorylation or unequal rates of transport of the dephosphorylated compounds.

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