Characterization of a Mutant *Bacillus subtilis* Adenylosuccinate Lyase Equivalent to a Mutant Enzyme Found in Human Adenylosuccinate Lyase Deficiency: Asparagine 276 Plays an Important Structural Role†

Jennifer Brosius Palenchar and Roberta F. Colman*

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

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ABSTRACT: Adenylosuccinate lyase, an enzyme catalyzing two reactions in purine biosynthesis (the cleavage of either adenylosuccinate or succinylaminoimidazole carboxamide ribotide), has been implicated in a human disease arising from point mutations in the gene encoding the enzyme. Asn^{276} of *Bacillus subtilis* adenylosuccinate lyase, a residue corresponding to the location of a human enzyme mutation, was replaced by Cys, Ser, Ala, Arg, and Glu. The mutant enzymes exhibit decreased V_{max} values (2−400-fold lower) for both substrates compared to the wild-type enzyme and some changes in the pH dependence of V_{max} but no loss in affinity for adenylosuccinate. Circular dichroism reveals no difference in secondary structure between the wild-type and mutant enzymes. We show here for the first time that wild-type adenylosuccinate lyase exhibits a protein concentration dependence of molecular weight, secondary structure, and specific activity. An equilibrium constant between the dimer and tetramer was measured by light scattering for the wild-type and mutant enzymes. The equilibrium is somewhat shifted toward the tetramer in the mutant enzymes. The major difference between the wild-type and mutant enzymes appears to be in quaternary structure, with many mutant enzymes exhibiting marked thermal instability relative to the wild-type enzyme. We propose that mutations at position 276 result in structurally impaired adenylosuccinate lyases which are assembled into defective tetramers.

Adenylosuccinate lyase (E.C. 4.3.2.2) is an enzyme participating in the purine biosynthetic pathway. Two distinct reactions are catalyzed: the cleavage of adenylosuccinate (SAMP)[1] to AMP and fumarate and the conversion of succinylaminoimidazole carboxamide ribotide (SAICAR) to aminoimidazole carboxamide ribotide (AICAR) and fumarate (*1*). The schematic structures of these compounds are shown in Figure 1. The reactions proceed by a β-elimination, involving the general acid/base residues His^{68} and His^{141} (*2*−*4*). (The residue numbers are those of *Bacillus subtilis* adenylosuccinate lyase.) Both His^{89} and Lys^{268} are considered to contribute to binding substrate (*5*, *6*), while Glu^{275} is an essential amino acid implicated in a catalytic dyad with His^{141} (*5*).

Adenylosuccinate lyase is a member of a superfamily of enzymes, including class II fumarase, δ-crystallin, carboxy-*cis*,*cis*-muconate lactonizing enzyme, aspartase, and argininosuccinate lyase. These enzymes all share high levels of structural similarity (*7*−*11*), despite low sequence identity. All are ∼200 kDa homotetramers and contain a 20-helix bundle in the center of the enzyme formed from the four subunits. Furthermore, each enzyme contains the signature sequence "SSxxPxK^{268}xNxxxxE^{275}" unique to the superfamily.

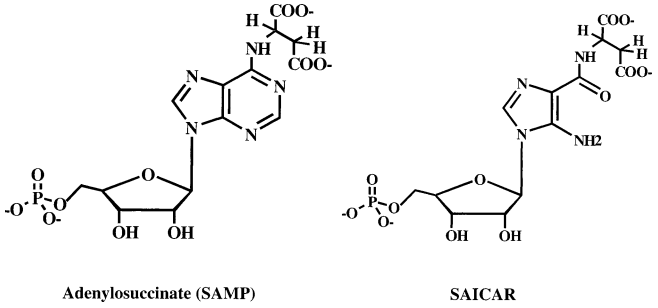

FIGURE 1: Schematic structural comparison of adenylosuccinate and SAICAR.

Our interest in studying this enzyme is largely due to its implication in a human disease. Adenylosuccinate lyase deficiency, first recognized in 1984 (*12*), often arises from a single point mutation in the gene encoding the enzyme and can result in mental retardation, muscle wasting, autistic features, and/or epilepsy. To date, 30 unique point mutations in the gene have been identified, in addition to 1 deletion (*13*). An additional characteristic of the disease is the presence of the normally undetectable dephosphorylated derivatives of SAMP and SAICAR, succinyladenosine (S-Ado) and succinylaminoimidazole carboxamide riboside (SAICA riboside), respectively, in the blood, urine, and cerebrospinal fluid. The ratio of the concentrations of S-Ado and SAICA riboside corresponds to the severity of the disease

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* To whom correspondence should be addressed. Phone: (302) 831-2973. Fax: (302) 831-6335. E-mail: rfcolman@chem.udel.edu.

[1] Abbreviations: AICAR, aminoimidazole carboxamide ribotide; SAICAR, succinylaminoimidazole carboxamide ribotide; SAMP, adenylosuccinate; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TAPS, *N*-tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid.

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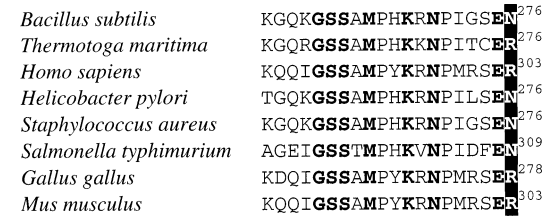

FIGURE 2: Sequence alignment of the signature sequence region of adenylosuccinate lyase from representative species, including human (*Homo sapiens*), chicken (*Gallus gallus*), and mouse (*Mus musculus*). Residues in bold are conserved among the adenylosuccinate lyases from all sources that have been sequenced to date (approximately 44). Contrasted are the residues corresponding to *B. subtilis* Asn^{276}. There is weak similarity at this position, with half the sources of the enzyme having an asparagine and the other half an arginine.

state presented (*14*): an S-Ado/SAICA riboside ratio of approximately 1 corresponds to a severe disease state, whereas a ratio of 2 or greater (values up to 5 have been reported) correlates with much milder symptoms. It has been postulated that SAICA riboside is the major toxic compound and that S-Ado protects tissues against its toxicity (*12*, *14*, *15*). Additionally, it is thought that mild disease symptoms correlate to a catalytically impaired enzyme, whereas severe symptoms correspond to a structurally impaired enzyme (*16*, *17*). Only a few biochemical studies have been carried out on the human enzyme (*17*−*19*); the instability of the human enzyme has been a major limitation in these studies. The results of these experiments pointed to differences in thermostability as accounting for the major distinction between the wild-type and mutant human enzymes. We seek to explore in greater depth the biochemical properties associated with mutations identified in the human disease.

Given the structural similarity among enzymes in this family and the relative instability of the human adenylosuccinate lyase, we have chosen to work with the more stable *B. subtilis* adenylosuccinate lyase as a model system. The crystal structure of adenylosuccinate lyase of *Thermotoga maritima* has been determined (*7*); the *B. subtilis* and *T. maritima* enzymes share 50% sequence identity and an additional 23% similarity at the amino acid level, while the *B. subtilis* and human adenylosuccinate lyases share 27% identity and 17% similarity. This sequence similarity has allowed us to construct models of both the human and *B. subtilis* enzymes based on the crystal structure of *T. maritima* adenylosuccinate lyase, as illustrated in Figure 8. These homology models assist in relating our results on the *B. subtilis* model system to the human enzyme.

One mutation of particular interest is R303C adenylosuccinate lyase (numbering according to the human enzyme). This mutation results in a mild disease state and yields an S-Ado/SAICA riboside ratio in patients' fluids ranging from 3 to 4 (*17*). The position corresponding to human Arg^{303} in the *B. subtilis* enzyme is Asn^{276}. Figure 2 shows the sequence alignment of the region surrounding Asn^{276} in the enzyme from representative species from bacteria to mammals. Among the 44 sources of adenylosuccinate lyase sequenced to date, there is weak similarity in this position (ClustalW), with an asparagine present in about half of the sources (including *B. subtilis*) and an arginine in the other half (including human). Accordingly, we constructed the N276C mutant *B. subtilis* enzyme to directly mimic the defective human enzyme and the N276R mutant *B. subtilis* adenylosuccinate lyase as a control in order to simulate the wild-type human enzyme.

Asn^{276} lies adjacent to the conserved signature sequence region (which includes Glu^{275}) and appears to be located just outside the active site. Thus, a series of mutations at position 276 not only allows us to biochemically characterize an enzyme in a human disease state but also affords the opportunity to probe the active site.

In addition, the biophysical studies of the wild-type and mutant enzymes also offer insight into our previously described complementation results (*5*). Adenylosuccinate lyase is a multimeric enzyme in which three subunits contribute residues to form the active site (Figure 8A). If inactive enzymes containing mutations to active site residues from different subunits are mixed together, a hybrid enzyme can be formed, thereby restoring partial activity. In order for this subunit complementation to occur, there must be dissociation of the tetramer and random reassociation of subunits. However, no species below the molecular weight of the tetramer has yet been observed for *B. subtilis* adenylosuccinate lyase. We now present evidence of a lower molecular weight species in both the wild-type and mutant enzymes. A preliminary version of some of this work has been presented (*20*).

EXPERIMENTAL PROCEDURES

Materials. Adenylosuccinate (SAMP), aminoimidazole carboxamide ribotide (AICAR), adenosine 5′-monophosphate, fumarate, MES, HEPES, TAPS, and imidazole were obtained from Sigma. The protein assay concentrate was from Bio-Rad. Oligonucleotides for site-directed mutagenesis and sequencing (nonfluorescent) were obtained from Bio-Synthesis. Fluorescent oligonucleotides for sequencing were from Li-Cor. All other chemicals were of reagent grade.

Site-Directed Mutagenesis. Mutations to the pBHis plasmid, encoding *B. subtilis* adenylosuccinate lyase, were generated through the use of the Stratagene QuikChange mutagenesis kit, as described previously (*3*, *21*). The following oligonucleotides and their complements were used to generate single amino acid changes at position 276, CCG ATT GGC TCT GAA TGC ATG ACA GGC ATG (Cys), CCG ATT GGC TCT GAA GCC ATG ACA GGC ATG (Ala), CCG ATT GGC TCT GAA AGC ATG ACA GGC ATG (Ser), CCG ATT GGC TCT GAA CGC ATG ACA GGC ATG (Arg), and CCG ATT GGC TCT GAA GAA ATG ACA GGC (Glu), and to construct the mutation at position 326, CTA CAT GCT GAA CAC CTT CTC CAA CAT C (Thr). The N276R/R326T double mutant was constructed by introducing a second mutation to the N276R cDNA. DNA sequencing confirmed the presence of the mutations and was performed at either the University of Delaware Cell Biology Core Facility using a Long-Readir 4200 DNA sequencer (Li-Cor) or at the Delaware Biotechnology Institute and University of Delaware Center for Agricultural Biotechnology using an ABI Prism model 377 DNA sequencer (PE Biosystems).

The protein, overexpressed in *Escherichia coli* strain BL21(DE3), contains a six-His tag at the N-terminus and was purified to homogeneity using Qiagen Ni-NTA−agarose as previously described (*2*, *21*). The purity of the resultant

enzymes was assessed by 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (*22*). Protein concentrations were determined either by the method of Bradford using wild-type adenylosuccinate lyase as the standard (*23*) or by absorbance at 280 nm using $E_{280}^{1\%} = 10.6$ (*2*).

Enzymatic Synthesis of SAICAR. Starting from AICAR (10 mM) and fumarate (150 mM), SAICAR was prepared enzymatically using adenylosuccinate lyase (0.4 mg/mL) in 50 mM HEPES, pH 7.0 (1 mL total volume). The reaction was allowed to proceed for 2 h, and the progress was monitored using PEI−cellulose plates with 1 M ammonium acetate, pH 6.40, as solvent (*18*). A Centricon-10 filtration device (Millipore) was used to remove the enzyme. The substrates were then applied to a DEAE-cellulose column (40 mL resin) equilibrated with 10 mM ammonium bicarbonate and eluted using a 10 mM (1 L) to 300 mM (1 L) ammonium bicarbonate linear gradient. A Dowex 50 column (Bio-Rad AG 50W-X4, 100−200 mesh, hydrogen form, 40 mL) was utilized for desalting the product. SAICAR was eluted using water. The final product was obtained in 13% yield.

Kinetics of B. subtilis Adenylosuccinate Lyases. Prior to any activity determination, each enzyme was incubated at a minimum concentration of 0.4 mg/mL in 20 mM potassium phosphate, containing 20 mM sodium chloride, pH 7.0, for 30 min at 25 °C. For adenylosuccinate, the activities of both the wild-type and mutant enzymes were assayed by monitoring the time-dependent decrease in absorbance at 282 nm as adenylosuccinate is converted to AMP and fumarate, using the difference extinction coefficient of 10000 M^{-1} cm^{-1} (*24*). For SAICAR, a decrease in absorbance was followed at 267 nm as SAICAR is converted to AICAR and fumarate, using the difference extinction coefficient of 700 M^{-1} cm^{-1} (*25*). Standard assay conditions were used in 50 mM HEPES, pH 7.0, at 25 °C, with 60 μM adenylosuccinate. For the activity ratios of SAMP and SAICAR, each substrate (90 μM) was used to ensure saturating conditions. In the direction of either AMP or AICAR formation, specific activity is defined as micromoles of substrate converted per minute per milligram of enzyme.

The k_{cat} and K_m values were determined for both SAICAR and adenylosuccinate by varying the substrate concentration. The data were analyzed by Lineweaver−Burk plots with error estimates obtained from the SigmaPlot software (SPSS Inc., Chicago, IL). Due to high absorbance at 282 nm, at adenylosuccinate concentrations greater than 150 μM, assays were conducted at 290 nm using a difference extinction coefficient of 4050 M^{-1} cm^{-1}.

The pH dependence of V_{max} was measured for the wild-type and Asn^{276} mutant enzymes, using the buffers MES (pH 6.3−6.9), HEPES (pH 6.8−8.0), and TAPS (pH 7.9−8.8), each constant in the basic species of the buffer (0.03 M). The effects of pH on enzyme activity were reversible over this pH range. The substrate concentrations for either SAMP or SAICAR in the assay were 300 or 100 μM, respectively. After the rate determination, the pH of each assay solution was measured. Data were analyzed using SigmaPlot software.

For the thermostability studies, each enzyme was preincubated for 30 min at 25 °C in 20 mM potassium phosphate, pH 7.0, containing 20 mM sodium chloride. After this incubation, the enzyme was transferred to a 42.5 °C water bath, and activity was assayed under standard conditions as a function of time.

Circular Dichroism Spectroscopy of Adenylosuccinate Lyase. Experiments were performed using a Jasco J-710 spectropolarimeter. Several spectra of the wild-type and each mutant enzyme were obtained over a concentration range from approximately 0.10 to 0.65 mg/mL, as determined by the method of Bradford. Prior to data acquisition, each sample was preincubated for 30 min at 25 °C in 20 mM potassium phosphate, containing 20 mM sodium chloride, pH 7.0. Ellipticity for each sample was measured in 0.2 nm increments from 250 to 200 nm, at room temperature. The samples were scanned five times and averaged, and the background buffer spectrum was subtracted out. The mean molar ellipticity was then calculated from the equation $[\theta] = \theta/10nCl$, where $[\theta]$ is the measured ellipticity (millidegrees), n is the number of amino acids in one subunit (437, including the six-His tag), C is the molar concentration of enzyme subunits, and l is the path length in centimeters (0.1 cm).

Molecular Weight Determination of the Wild-Type and Asn^{276} Mutant Enzymes. A miniDAWN laser photometer (Wyatt Technology Corp., Santa Barbara, CA) was used to determine the molecular weights of the wild-type and Asn^{276} mutant enzymes. The molecular weight of each enzyme was determined at several different protein concentrations ranging from approximately 0.10 to 0.35 mg/mL (as determined by the method of Bradford after the light scattering experiments). The enzyme was preincubated at 25 °C in 20 mM potassium phosphate, pH 7.0, also containing 20 mM sodium chloride. Data were collected at room temperature, using a laser wavelength of 690 nm, and were subsequently analyzed using ASTRA software for Windows. The operation and theory of this instrument may be found in ref *26*. Lysozyme samples of known molecular weight and concentration were used for instrument normalization.

Homology Modeling of Human Adenylosuccinate Lyase. The homology model of the human adenylosuccinate lyase was created using the crystal structure of *T. maritima* adenylosuccinate as the template (PDB code1c3c). This model was created by the same methods used for the homology model of *B. subtilis* adenylosuccinate lyase as described previously (*5*), with the exception that the 26 additional N-terminal amino acids of the human enzyme were truncated prior to modeling.

RESULTS

Purity of the Asn^{276} Mutant Adenylosuccinate Lyases. Asn^{276} of *B. subtilis* adenylosuccinate lyase occurs at the position corresponding to position 303 in the human enzyme, which is mutated in one form of human adenylosuccinate lyase deficiency (*17*, *27*). We replaced Asn at position 276 by several other amino acids: cysteine (the mutation found in the human disease), alanine (to test the importance of decreasing the size of the side chain), serine (a residue similar in size to cysteine but with different functionality), arginine (the corresponding wild-type human residue), and glutamate (to evaluate the effect of a negative charge at position 276). A double mutant, N276R/R326T, was also constructed. This double mutation was intended to simulate, in the *B. subtilis* enzyme, the local environment of the wild-type human enzyme, since the wild-type human enzyme has an Arg at

Table 1: Summary of the Wild-Type and Asn[276] Adenylosuccinate Lyase Kinetic Parameters in the Direction of AMP Formation[a]

enzyme	V_{max}[b] (μmol min^{-1} mg^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
wild type	1.56 ± 0.19	3.46 ± 0.44	1.30 ± 0.16	3.75 × 10^5
N276C	0.63 ± 0.04	10.4 ± 0.99	0.52 ± 0.04	0.51 × 10^5
N276A	0.17 ± 0.01	7.40 ± 0.47	0.14 ± 0.005	0.12 × 10^5
N276S	0.16 ± 0.01	5.17 ± 0.78	0.13 ± 0.005	0.25 × 10^5
N276R	0.020 ± 0.0006	4.17 ± 0.39	0.017 ± 0.0006	0.04 × 10^5
N276E	0.0037 ± 0.003	nd[c]	nd[c]	nd[c]
N276R/R326T	0.38 ± 0.01	2.60 ± 0.22	0.31 ± 0.01	1.21 × 10^5

[a] The activities and K_ms for these enzymes were determined spectrophotometrically at 25 °C in 50 mM HEPES buffer, pH 7.0, in the presence of adenylosuccinate, as described in Experimental Procedures. [b] V_{max} is defined as micromoles of substrate converted per minute per milligram of enzyme. The lower limit of detectable activity is approximately 5 × 10^{-5} μmol min^{-1} mg^{-1}. [c] nd = not determined.

position 276 and a Thr at position 326. The numbers correspond to the *B. subtilis* enzyme. The purity of the resultant enzymes was assessed by SDS−PAGE gels (data not shown). Each enzyme exhibited a single subunit band with the expected subunit molecular weight of about 50000.

Kinetic Parameters of the Asn[276] Mutant Enzymes, N276R/R326T, and the Wild-Type Enzyme. All of the mutant enzymes constructed, with the exception of N276E, had sufficient activity to characterize kinetically. The kinetic data at pH 7.0 in the direction of AMP formation for the wild-type and Asn[276] mutant enzymes and N276R/R326T adenylosuccinate lyase are summarized in Table 1. Compared to a wild-type value of 1.56 μmol of adenylosuccinate min^{-1} mg^{-1}, the V_{max} values of the N276C, N276A, N276S, and N276R/R326T enzymes are only modestly reduced at 0.63, 0.17, 0.16, and 0.38 μmol of adenylosuccinate min^{-1} mg^{-1}, respectively. However, the activities of the N276R and N276E adenylosuccinate lyases, 0.02 and 0.004 μmol of adenylosuccinate min^{-1} mg^{-1}, respectively, are considerably lower than that of the wild-type enzyme. It was surprising that the N276R enzyme is so low in activity since arginine is present in the human enzyme at this position. However, the *B. subtilis* enzyme has another arginine at position 326 which is relatively close to position 276 in this enzyme (see Discussion); electrostatic repulsion between the two arginines may account for the low activity of the N276R *B. subtilis* enzyme. In the human enzyme, the amino acid at the position corresponding to position 326 is threonine. The N276R/R326T mutant enzyme, intended to better simulate wild-type human adenylosuccinate lyase, does indeed have significantly more activity relative to the N276R enzyme (Table 1). The K_m values for adenylosuccinate are not appreciably altered from that of the wild type in any of the mutant enzymes. Since most of the replacements at position 276 yield enzymes with substantial activity, the primary role of Asn[276] is not catalytic.

pH Dependence of V_{max} for the Wild-Type and Mutant Adenylosuccinate Lyases. More significant differences between wild-type adenylosuccinate lyase and the mutant enzymes became apparent upon determination of the dependence of velocity on pH. An extrapolated V_{max} in the direction of AMP formation was determined for each enzyme from pH 6.3 to pH 9.0. K_ms for adenylosuccinate were determined across the pH range, and the observed rates were extrapolated to V_{max} using the equation $V_{max} = v_{obs}(1 + K_m/[substrate])$. All enzymes yielded a bell-shaped pH−V_{max} profile, as represented by the wild-type enzyme (Figure 3, ■), and the pK values were determined as previously described (*6*). A summary of pK_1 and pK_2 values is in Table 2. The value of pK_1 does not vary appreciably between the wild-type and mutant enzymes. However, pK_2 is more affected by the mutations at position 276; this value is decreased in most of these mutant enzymes tested.

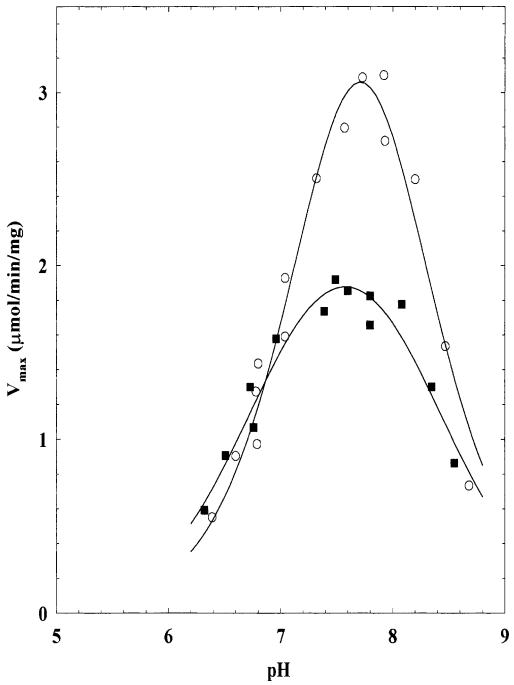

FIGURE 3: pH−V_{max} rate profiles for wild-type adenylosuccinate lyase with (○) SAICAR and (■) adenylosuccinate. The data are expressed in terms of specific activity and were extrapolated to V_{max}. With SAICAR, pK_1 and pK_2 have values of 7.41 ± 0.10 and 8.01 ± 0.12, respectively. pK_1 and pK_2 with adenylosuccinate are 6.78 ± 0.08 and 8.37 ± 0.09, respectively.

Table 2: pK Values Obtained from pH−V_{max} Profiles for Wild-Type and Mutant Adenylosuccinate Lyases[a]

enzyme[b]	pK_1	pK_2
wild type	6.78 ± 0.08	8.37 ± 0.09
N276C	7.03 ± 0.13	8.34 ± 0.14
N276A	7.14 ± 0.17	7.41 ± 0.18
N276S	6.91 ± 0.10	7.83 ± 0.11
N276R/R326T	6.74 ± 0.08	7.97 ± 0.08

[a] These pK values were obtained at 25 °C, as described in the Experimental Procedures, from plots exemplified by Figure 3. [b] N276E and N276R lacked sufficient activity to accurately determine the pK values.

Characterization of Wild-Type Adenylosuccinate Lyase with SAICAR. Adenylosuccinate lyase is unique in the purine biosynthetic pathway in that it catalyzes two discrete reactions involving different substrates: adenylosuccinate and SAICAR. The question may be raised as to whether the same residues are involved in catalysis of both substrates. We therefore measured the kinetic parameters of wild-type adenylosuccinate lyase with either adenylosuccinate or SAICAR. The results are summarized in Table 3. V_{max}, K_m, and k_{cat} are all slightly higher with SAICAR than adenylosuccinate, indicating that the enzyme is about equally effective in catalyzing the two reactions.

Table 3: Kinetic Parameters for Wild-Type Adenylosuccinate Lyase with Adenylosuccinate and SAICAR[a]

substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1}s^{-1})
SAMP	1.56 ± 0.19	3.46 ± 0.44	1.30 ± 0.16	3.75×10^5
SAICAR	1.94 ± 0.10	6.40 ± 0.47	1.61 ± 0.08	2.51×10^5

[a] The characterization of wild type in the presence of either SAMP or SAICAR was carried out at 25 °C in 50 mM HEPES buffer at pH 7.0.

Table 4: Comparison of Adenylosuccinate and SAICAR Activities for Wild-Type and Mutant Adenylosuccinate Lyases[a]

enzyme	SAMP (μmol min^{-1} mg^{-1})	SAICAR (μmol min^{-1} mg^{-1})	SAICAR/SAMP
wild type	1.56	1.94	1.27
N276C	0.44	0.36	0.82
N276R/R326T	0.23	0.30	1.30
N276A	0.13	0.28	2.15
N276S	0.083	0.17	2.05
N276R	0.012	0.045	3.75
N276E	0.003	0.027	9.00

[a] Activities were determined at 25 °C in 50 mM HEPES, pH 7.0, in the presence of either 90 μM SAICAR or SAMP (concentrations which are saturating for the substrate sites).

A pH$-V_{max}$ profile was also determined using either adenylosuccinate or SAICAR (Figure 3). Utilizing SAICAR as the substrate, pK_1 and pK_2 values of 7.41 ± 0.10 and 8.01 ± 0.12, respectively, were calculated. For comparison, values for pK_1 and pK_2 are 6.78 ± 0.08 and 8.37 ± 0.09, respectively, as obtained with adenylosuccinate as substrate. Both pK_1 and pK_2 are significantly different for the two substrates, indicating that in the enzyme$-$substrate complex the pK values are influenced by the substrate. Visually inspecting the two pH$-$rate profiles, at the peak activities, SAICAR activity ($V_{max} \sim 3.05$ μmol min^{-1} mg^{-1}) is approximately 1.6 times greater than that of SAMP ($V_{max} \sim 1.90$ μmol min^{-1} mg^{-1}). However, using the equation $V_{max} = V_o/(1 + 10^{pK_1-pH} + 10^{pH-pK_2})$, the pH-independent V_{max} values (V_o) were determined for SAICAR and SAMP as 6.13 ± 0.89 and 2.48 ± 0.160 μmol min^{-1} mg^{-1}, respectively.

SAICAR/SAMP Ratios of the Wild-Type and Asn^{276} Mutant Enzymes. Previous reports have indicated that in some cases of adenylosuccinate lyase deficiency the two activities of the enzyme are affected differentially (*12*, *14*). To evaluate whether the mutations at position 276 affect the ability of the enzymes to catalyze the reaction of one substrate more markedly than the other, the specific activity was determined at pH 7.0 for each mutant enzyme, using both SAICAR and adenylosuccinate as substrates. The ratio of these activities at 90 μM SAICAR and SAMP is compared in Table 4. These concentrations are high relative to the K_ms for SAMP (Table 1) and for SAICAR. K_m values for SAICAR were determined for the mutant enzymes and found to be 9.58 ± 1.34 μM (N276C), 4.42 ± 1.21 μM (N276A), 8.43 ± 1.00 μM (N276S), and 12.60 ± 1.89 μM (N276R/R326T). These K_m values are not appreciably different from that of the wild-type enzyme (6.40 μM), as shown in Table 3. The N276R and N276E enzymes did not have sufficient activity to determine a K_m for SAICAR because of the lower sensitivity of the assay for the SAICAR as compared to the SAMP reaction.

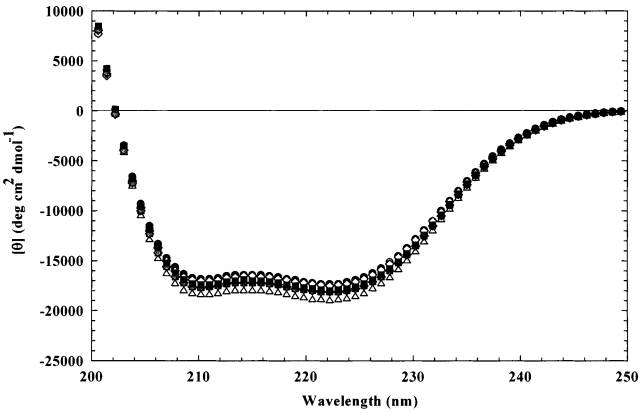

FIGURE 4: Circular dichroism spectroscopy of the wild-type and N276X mutant enzymes at pH 7.0, room temperature, and 0.45 mg/mL protein concentration for each enzyme: (○) wild type, (■) N276A, (△) N276C, (●) N276R, (◇) N276S, (◆) N276E, and (■) N276R/R326T.

The wild-type enzyme has a ratio of specific activities of SAICAR/SAMP of 1.27. The ability of the mutant enzymes to catalyze the reactions of both SAMP and SAICAR is impaired; however, the decrease in the rate of cleavage of adenylosuccinate is generally greater than that of SAICAR. The ratio of the SAICAR to adenylosuccinate activity is only moderately elevated from wild type in the N276A and N276S enzymes (to 2.15 and 2.05, respectively). However, the activity ratio is increased to 3.75 in the N276R enzyme and to 9.00 in the N276E enzyme. Interestingly, the double mutant N276R/R326T enzyme has an activity ratio similar to that of the wild-type enzyme, even though there is a decrease in the enzyme-catalyzed rates of reactions of both substrates. It appears that certain mutations at position 276 compromise the ability of the enzyme to catalyze the adenylosuccinate reaction to a greater extent than the SAICAR reaction.

Circular Dichroism Spectroscopy of the Wild-Type and Mutant Enzymes. Since the kinetic parameters of most of the mutant enzymes are not appreciably altered relative to the wild-type enzyme, circular dichroism was utilized to determine whether the primary effect of these mutations is on the secondary structure of the enzyme. As shown in Figure 4, the spectra of the mutant adenylosuccinate lyases at room temperature, each at a concentration of 0.45 mg/mL, are virtually superimposable upon that of the wild-type enzyme. These results indicate that their secondary structures are not significantly different from that of the wild type. All of the enzymes exhibit minima at 208 and 222 nm, typical of proteins containing appreciable amounts of α-helix.

Wild-Type Adenylosuccinate Lyase: A Concentration Dependence of Molecular Weight, Secondary Structure, and Activity. Adenylosuccinate lyase is known to be a tetramer in which three subunits contribute to each active site. Since we have previously shown that two different inactive mutant adenylosuccinate lyases exhibit complementation and activation, the enzyme must undergo reversible dissociation. Yet, only the tetramer has been observed, using both gel filtration and native gel electrophoresis. Light scattering affords us the chance to examine the molecular weight of the enzyme at much lower protein concentrations than are possible with the aforementioned techniques. Thus, we used light scattering to evaluate whether a smaller protein species is present.

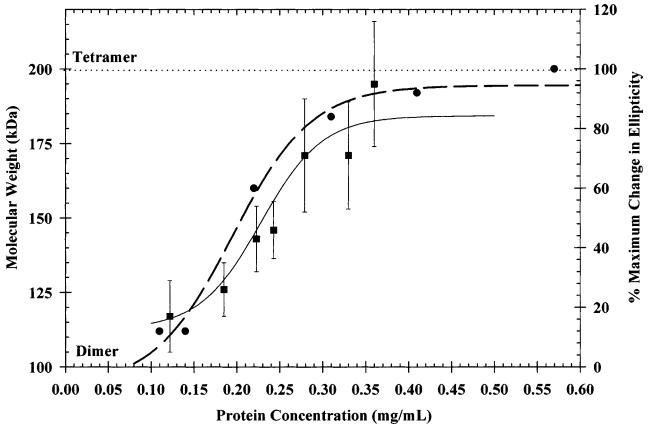

FIGURE 5: Overlay of the protein concentration dependence of molecular weight and secondary structure for wild-type adenylosuccinate lyase as determined by light scattering and circular dichroism, respectively. The weight-average molecular weights plotted (■, —) represent a sample run. The curve shown (solid line) is not theoretical; rather it is intended only to indicate the trend of the data. The right axis represents the percent maximum change in ellipticity at 222 nm (●, ---). Typical values for $[\theta]$ minima were in the range of -9000 deg cm^2 dmol^{-1}, and maximal values were approximately -15000 deg cm^2 dmol^{-1}. The final enzyme concentrations for both sets of experiments were determined by the method of Bradford (*4*).

Enzyme concentrations, ranging from 0.10 to 0.35 mg/mL, were examined. For the first time, a concentration dependence of molecular weight was observed for *B. subtilis* adenylosuccinate lyase. Representative data for the wild-type enzyme is shown in Figure 5. At the lowest protein concentrations, the major species appears to be the enzyme dimer, with the tetramer forming at higher concentrations. If it is assumed that the predominant equilibrium is between the tetramer and dimer, a K_d can be calculated using the equation $K_d = [\text{dimer}]^2/[\text{tetramer}]$ (*28*). At every measured protein concentration (in milligrams per milliliter), the weight-average molecular weight was also determined. Since the molar concentration of the total enzyme is assumed to be equal to the sum of the molar concentrations of the dimer and tetramer, the concentration of each of these species can be calculated from the experimental data and the known molecular weights of the dimer (100614) and tetramer (201228). A K_d value of 2.52×10^{-6} M was estimated for the wild-type enzyme.

To test the possibility that substrate induces tetramer formation, experiments were performed in which the enzyme was incubated with varying amounts of substrate, i.e., 1−5 mM AMP and 10 mM AMP + 150 mM fumarate. The protein concentration dependence of molecular weight was not significantly different from that of the enzyme without substrate, indicating that substrate does not influence tetramer formation.

Circular dichroism was used to probe for a concentration dependence of secondary structure over the range of approximately 0.10−0.65 mg/mL (Figure 5). The $[\theta]_{222nm}$ at the lowest enzyme concentration (-9000 deg cm^2 dmol^{-1}) is only 60% of the $[\theta]_{222nm}$ at the highest concentration (-15000 deg cm^2 dmol^{-1}). The results are expressed as the percent maximum change in ellipticity [defined as $100(\theta - \theta_{min})/(\theta_{max} - \theta_{min})$], where θ is the value measured at a particular protein concentration. The change in secondary structure clearly correlates well with the change in molecular weight.

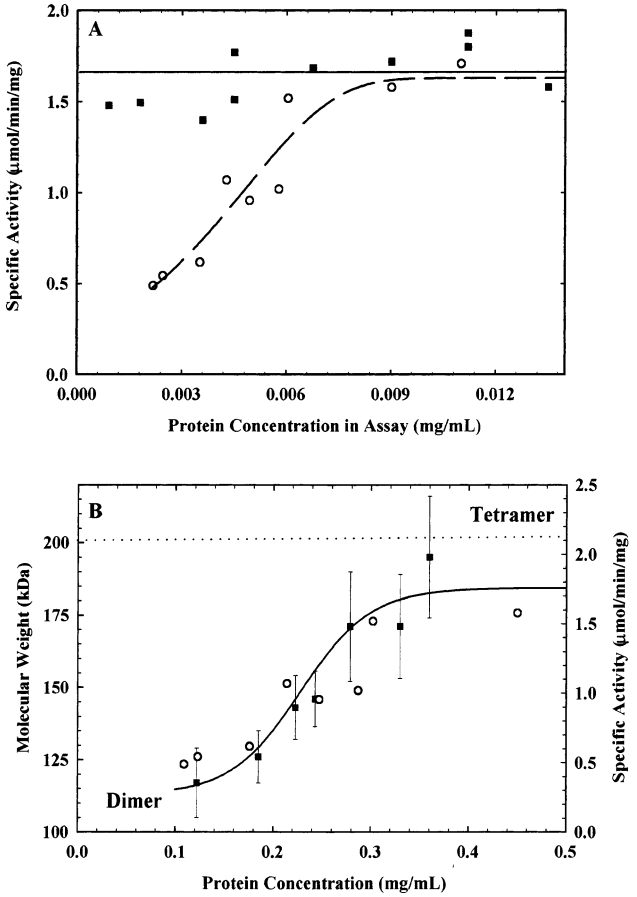

FIGURE 6: (A) Protein concentration dependence of ASL specific activity. (■) represents a protein sample which has been incubated at 0.45 mg/mL (25 °C, for 30 min) and then diluted rapidly into the assay to give the final assay protein concentration indicated. Alternately, several protein samples were incubated across a range of concentrations (0.10−0.45 mg/mL) and then diluted into the assay (○). At higher protein concentrations (~0.30 mg/mL), the same specific activity is measured for the enzymes from the two experiments. Shown in (B) is the correlation between the observed average molecular weight (■) and the enzyme specific activity (○) when the enzyme is incubated across a protein concentration range.

The effect of protein concentration on the catalytic activity was also evaluated. Two different types of experiments were conducted. In the first, the wild-type enzyme was preincubated at 25 °C for 30 min at concentrations ranging from 0.1 to 0.55 mg/mL enzyme (similar to those used for the light scattering experiments), and equal volumes (20 μL) were assayed for 2 min under standard conditions in a cuvette containing a total volume of 1000 μL. The enzyme specific activity increased with increasing protein concentration: at the lowest concentrations, the specific activity was 0.5 μmol min^{-1} mg^{-1}, but at preincubation protein concentrations at or above 0.40 mg/mL, the specific activity reached a constant maximum (Figure 6A, ○). Figure 6B illustrates how the increasing specific activity parallels the increased molecular weight observed over the protein concentration range examined.

In a separate experiment, the enzyme was preincubated at 25 °C for 30 min at a protein concentration of 0.45 mg/mL. Various volumes, to yield final protein concentrations in the assay mixture that correspond to the assay concentra-

Table 5: Dissociation Constants for Tetramer−Dimer Equilibrium for Wild-Type and Mutant Enzymes[a]

enzyme	K_d (μM)	enzyme	K_d (μM)
wild type	2.52 ± 1.63	N276R	0.32 ± 0.36
N276S	0.91 ± 0.57	N276E	0.17 ± 0.11
N276A	0.64 ± 0.38	N276C	0.13 ± 0.03

[a] Protein samples, ranging in concentration from 0.1 to 0.35 mg/mL, were incubated in 20 mM KPO_4, containing 20 mM NaCl, pH 7.0, at 25 °C. Molecular weight determination via light scattering was performed at room temperature. Representative data are shown in Figure 5.

tions evaluated above, were then rapidly diluted into the assay solution, and the activity was monitored. In this case, the specific activity remained at a constant maximal level even though the protein concentration varied in the assay cuvette (Figure 6A, ■). As an additional experiment, the enzyme was preincubated at the lower concentration of 0.1 mg/mL, and larger volumes were added to the assay cuvette to correspond to the entire protein range assayed; the specific activity remained low over the entire protein concentration range studied (data not shown). The results from these two sets of experiments suggest that the equilibrium between the dimer and tetramer is established slowly relative to the time period of the assay (2 min) and that the protein preincubation concentration determines both the specific activity and the percent enzyme in the tetrameric form. These results indicate the relatively slow formation of the active tetramer (occurring best at high protein concentrations) which does *not* dissociate rapidly once diluted into the assay. At lower protein concentrations, the weight-average molecular weight is still slightly greater than that of dimer; it is likely that the observed activity at these concentrations reflects that of the small amount of tetramer present. These data also highlight the need to carefully consider protein concentration in the design of experiments involving adenylosuccinate lyase. For the measurement of the kinetic parameters and pH−V_{max} rate profiles reported in this paper, the enzyme was incubated at a concentration of at least 0.4 mg/mL to ensure its being in the most active form.

Molecular Weight Determination of the Asn^{276} and N276R/R326T Enzymes. Given the observed protein concentration dependence of specific activity of the wild-type enzyme, it was important to ascertain whether the state of aggregation was altered in the mutant enzymes. The mutant enzymes also exhibited a protein concentration dependence of molecular weight, reaching a maximum of approximately 200 kDa at high protein concentration. K_ds for N276A, N276C, N276E, N276R, and N276S adenylosuccinate lyases were determined by light scattering under the same conditions used for wild type (Table 5). The K_ds for the mutant enzymes are all somewhat lower than that of wild type, which is reflected in a higher observed M_w (weight-average molecular weight) for the mutant as compared to the wild-type enzyme at the same protein concentration. These results imply that, in the Asn^{276} mutant enzymes, the dimer−tetramer equilibrium favors tetramer more than in the case of the wild-type enzyme. (Light scattering was not utilized for N276R/R326T because of the presence of a small amount of aggregated protein.)

Thermostability Studies of the Wild-Type and Asn^{276} Mutant Adenylosuccinate Lyases. Because (in the crystal structure) Asn^{276} appears to be located just outside the active site, we considered that mutations at that position might affect enzyme structure and stability more than catalytic activity. To explore this possibility, thermostability studies of the enzyme at 42.5 °C were conducted (Figure 7A). This temperature was chosen because it provided the greatest difference between the enzyme samples over a relatively short time period. (At 25 °C, the temperature at which most other experiments were carried out, the wild-type and mutant enzymes were stable for many hours.) Several of the mutant enzymes (N276R, N276A, and N276S) precipitated within the time period studied, indicating their relative instability. Both the wild-type enzyme and N276C adenylosuccinate lyase were not fully stable, having rate constants for loss of activity of 8.17×10^{-3} min^{-1} and 2.08×10^{-3} min^{-1}, respectively. The introduction of a second mutation, N276R/R326T, countered the instability introduced by the N276R mutation; this enzyme was completely stable over the entire time period studied. Thus, both N276C and the double mutant N276R/R326T were more stable than the wild-type enzyme.

Because 10% glycerol appears to stabilize the tetrameric form of the wild-type adenylosuccinate lyase (Palenchar and Colman, unpublished data), the above experiments were then repeated for all enzymes in the presence of 10% glycerol (Figure 7B). All enzymes were markedly more stable with

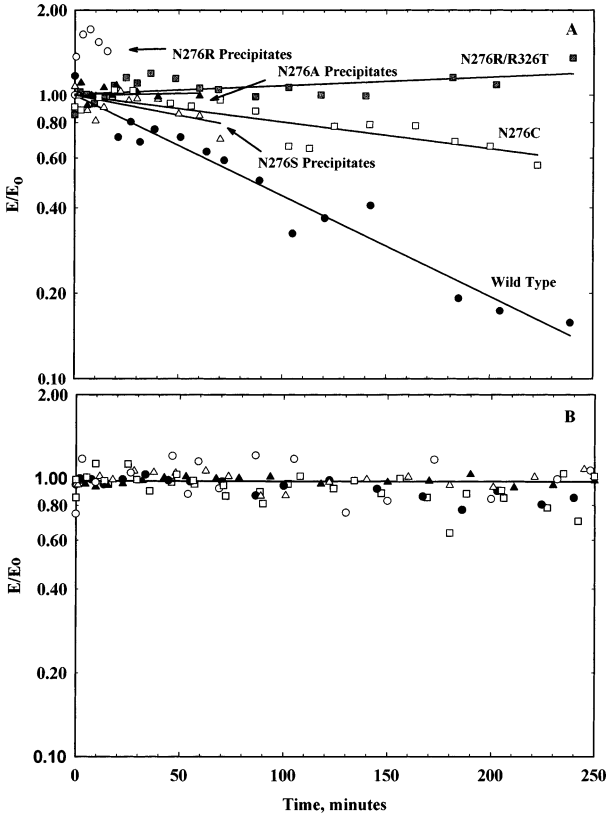

FIGURE 7: Thermostability studies of the Asn^{276} mutant enzymes and wild-type ASL at 42.5 °C. (A) The following proteins are shown: (●) wild type, (○) N276R, (□) N276C, (△) N276S, (▲) N276A, and (■) N276R/R326T. Wild-type enzyme exhibits a rate constant (k) of 8.17×10^{-3} min^{-1}; for N276C $k = 2.08 \times 10^{-3}$ min^{-1}, and for N276S $k = 3.24 \times 10^{-3}$ min^{-1}. The enzyme activities are expressed as E/E_0 (observed activity/initial activity). (B) The above experiment was repeated in the presence of 10% glycerol. All proteins were stabilized over the time period studied, with the exception of N276R, which was turbid throughout the experiment after 20 min but still retained full activity.

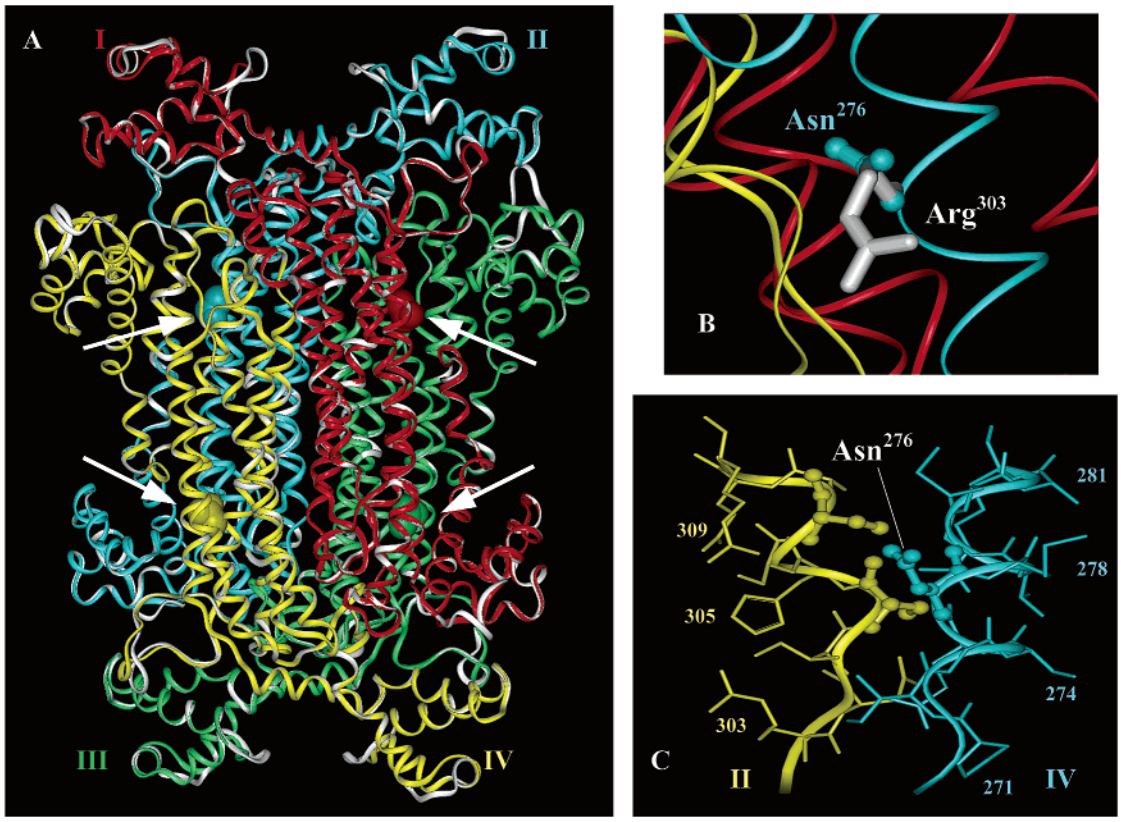

FIGURE 8: (A) Overlaid models of the *B. subtilis* adenylosuccinate lyase (multicolored subunits) and the human enzyme (white). The only significant structural deviation is found at the outer regions of the enzyme, away from residue 276. The white arrows indicate the position of Asn^{276} displayed in *B. subtilis* adenylosuccinate lyase. (B) Superimposed *B. subtilis* (cyan, red, and yellow) and human adenylosuccinate lyases in the region of *B. subtilis* position 276. Only residues Asn^{276} (*B. subtilis*, cyan) and Arg^{303} (human, white) are shown to emphasize that the two residues are spatially conserved. These residues completely overlap through the γ-carbon of arginine and the carbonyl carbon of asparagine. (C) Subunit interface region containing the Asn^{276} residue. Asn^{276} is shown in cyan, and the two subunits are in cyan and yellow (corresponding to the tetramer color scheme). The closest interaction between the side chain of Asn and the other subunit is only 3.3 Å.

added glycerol. Only the N276R enzyme became turbid, and some precipitation was observed; however, if each aliquot of this enzyme was evenly suspended prior to assay, it still retained full activity.

These results are likely to reflect the differing equilibria between the dimer and tetramer for the wild-type and mutant enzymes. If the dimer is more susceptible to thermal denaturation, the relative instability of the wild type can be understood. The mutant enzymes may not assemble *correctly* to the tetramer, thus accounting for the increased aggregation and precipitation. The addition of glycerol presumably stabilizes and shifts the equilibrium toward the tetramer in the case of even the wild-type enzyme, accounting for the increased stability. The mutant enzymes, in the presence of glycerol, are probably less likely to aggregate (and hence precipitate), which is consistent with our observed results.

DISCUSSION

The importance of Asn^{276} in adenylosuccinate lyase of *B. subtilis*, a residue corresponding to a mutation found in human adenylosuccinate lyase deficiency, was evaluated through site-directed mutagenesis. However, in the course of evaluating the biophysical properties of the mutant enzymes, significant new properties of the wild-type enzyme were discovered. A protein concentration dependence of molecular weight for *B. subtilis* adenylosuccinate lyase was observed for the first time. At lower protein concentrations (~0.10 mg/mL), the majority of the enzyme appears to exist as a dimer, with an average molecular weight of approximately 125000. As the concentration is increased, the molecular weight levels off at a tetrameric molecular weight. This observation has important implications for the subunit complementation experiments (*4*−*6*). In order for complementation to occur, the enzyme must dissociate to a lower molecular weight species, i.e., a dimer and/or monomer. This is the first direct determination for adenylosuccinate lyase of the molecular weight of a naturally occurring lower molecular weight species. [Woodward and Braymer (*25*) did observe in *Neurospora* adenylosuccinate lyase a smaller species, 27 kDa, which was termed a "monomer"; however, this was likely an artifact of proteolytic cleavage occurring during enzyme purification.]

We here show that the wild-type adenylosuccinate lyase secondary structure also exhibits a dependence on protein concentration. The overall shape of the circular dichroism spectra over a range of protein concentration varies in its intensity rather than in its general shape (which is typical of α-helix, with minima at 208 and 222 nm). Formation of the tetramer as the protein concentration increases likely shields the central helical domain (Figure 8A) from the solvent, probably resulting in an increased amount of α-helix. It is notable that this concentration dependence of secondary structure corresponds well with that observed for molecular weight (Figure 5).

In this study, we also characterized the kinetics of wild-type adenylosuccinate lyase with its second substrate, SAICAR. The V_{max} for SAICAR is only minimally higher than that for adenylosuccinate at pH 7.0, and the K_m values for the two substrates are not significantly different. Thus, the enzyme appears to handle both substrates approximately equally under these conditions. That the kinetic parameters for adenylosuccinate and SAICAR are comparable at pH 7.0 is not surprising given the structural similarity of the two compounds (Figure 1). It is reasonable that the enzyme might utilize the same residues to catalyze both elimination reactions. However, the pH−V_{max} profiles are distinct for the two substrates: SAICAR has pK_1 and pK_2 values of 7.41 and 8.01, respectively, compared to 6.78 and 8.40 for adenylosuccinate. It is thought that the pK_2 of the wild-type enzyme reflects the deprotonation of both His^{68} and His^{89} and has a ΔH value corresponding to that of a histidine (*6*). It is probable that these active site residues are modestly adjusted in order to accommodate the alternate substrates and, thus, are located in slightly different environments in the two enzyme−substrate complexes; this is reflected in the minor differences observed in pK_2. The enzyme or substrate group associated with pK_1 has yet to be determined but is a neutral acid since the ΔH for ionization was previously shown to be close to zero (*6*); it has been postulated to be due to dissociation of a proton from the substrate's phosphoryl group or a carboxyl in the enzyme−substrate complex. Despite numerous mutations made at various positions in the active site, pK_1 has not previously been altered. The observed differences in p*K* values between the substrates may prove useful in elucidating the identity of the ionizable group reflected in pK_1.

We have now examined the properties of mutant enzymes resulting from a series of substitutions for Asn^{276} of *B. subtilis* adenylosuccinate lyase. Asn^{276} was selected for study via site-directed mutagenesis because it is not only in the position corresponding to a residue in the human enzyme that has been implicated in the human disease state but it also neighbors Glu^{275}, a residue previously shown to be essential for enzyme activity (*5*). We consider it reasonable to use the *B. subtilis* enzyme as a model system for elucidating the biochemical basis of human adenylosuccinate lyase deficiency. These two enzymes align well in the region of interest, so we are confident that Asn^{276} is the corresponding *B. subtilis* residue. Furthermore, a model of the human enzyme was constructed, and if the *B. subtilis* enzyme and the human enzyme models are superimposed (shown in Figure 8A), the overall structures of the two are highly similar, with the minor deviations being located away from the area being studied. Importantly, Asn^{276} of *B. subtilis* and the corresponding Arg^{303} of human are spatially conserved and superimpose as shown in Figure 8B. The backbone and side chain atoms of these two residues completely superimpose through the γ-carbon of the arginine and the carbonyl carbon of the asparagine. Figure 8C illustrates further the importance of this residue: it is located at the intersection of three subunits, but Asn^{276} from the cyan subunit most closely interacts (less than 3.5 Å) with the subunit shown in yellow. This positioning gives Asn^{276} the potential to play a role in mediating subunit interactions and/or interfering with subunit interactions if an amino acid substitution is made. We consider structure to be critical in this case, not necessarily complete amino acid conservation. Additionally, we were motivated to use the *B. subtilis* enzyme as a model system by the reported instability of the recombinant human enzyme (*17*). (Because the human enzyme was unstable, the human adenylosuccinate lyase was isolated as a fusion protein with thioredoxin, and due to the enzyme's instability, fresh protein had to be purified for each experiment.)

Initial kinetic characterization of the *B. subtilis* Asn^{276} mutant enzymes revealed that all of the enzymes have decreased V_{max} values for adenylosuccinate as substrate, which vary from 2- to 400-fold lower than that of the wild-type enzyme. On the basis of these activities, it appears that a charged residue (i.e., glutamate or arginine) is not well tolerated at this location; rather a neutral polar residue (such as cysteine) is preferred. However, the K_m values for adenylosuccinate remain relatively unchanged in all of the mutant enzymes, indicating that their affinity for substrate is not significantly altered as a result of a mutation at this position. The pH−rate profiles (with adenylosuccinate as substrate) revealed that pK_1 is essentially unaltered, but pK_2 decreases somewhat (up to 1 p*K* unit in the N276A enzyme). These observed shifts in p*K* may best be interpreted in terms of subtle changes in the environment of His^{68} and His^{89}, resulting from effects on subunit interaction. The active site of this enzyme is assembled from three different subunits. If the subunit interface at the active site is even slightly distorted, the local environment may be modulated and the distances between critical residues may change, resulting in shifted p*K* values. All mutant enzymes with replacements at position 276 have essentially unaltered substrate affinity, and most retain appreciable activity; these observations support the idea that the role of this particular residue is not catalytic.

It was initially troubling to see that the N276R enzyme, corresponding to the wild-type human enzyme, had one of the lowest specific activities. However, a comparison of both the human and *B. subtilis* enzyme models in this region offers an explanation for the reduced activity of N276R enzyme. In the *B. subtilis* enzyme, if position 276 is occupied by an arginine (Figure 9A), there are numerous unfavorable interactions, particularly with another arginine located at position 326. In the human enzyme, though, the position corresponding to *B. subtilis* position 326 is a threonine (Figure 9B). This arrangement eliminates the electrostatic repulsion associated with two arginines in close proximity. Hence, we constructed the N276R/R326T double mutant enzyme with the goal of converting the *B. subtilis* enzyme into a more human-like enzyme, alleviating the charge repulsion and, accordingly, increasing enzyme activity. Indeed, the double mutant *B. subtilis* enzyme possesses considerably more activity than does the single N276R mutation; the activity of the N276R/R326T enzyme is roughly 20 times higher than that of the N276R enzyme. These results not only support the use of the model of human adenylosuccinate lyase but demonstrate how the two enzymes may be "interconverted" by a relatively small number of amino acid substitutions. The relationship between Asn^{276} and Arg^{326} of *B. subtilis* appears to be a well-conserved coexistence among the adenylosuccinate lyases sequenced (44 to date). In the enzymes where the position corresponding to position 276 is occupied by Asn, the majority have a positively charged residue (Arg or Lys) at position 326, while

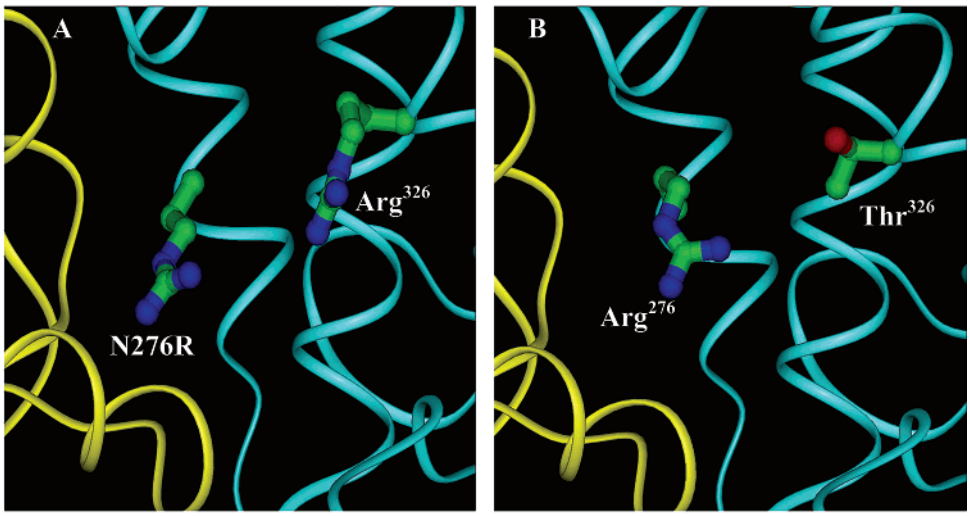

FIGURE 9: (A) If *B. subtilis* ASL contains the N276R substitution, there is unfavorable interaction with Arg^{326}. An examination of all possible rotamers for Arg at position 276 revealed that there are no conformations in which interactions are favorable. In most rotamers, the guanido groups of the two arginines are interacting too closely. (B) The human enzyme residues corresponding to *B. subtilis* positions 276 and 326 are shown. Although there is an Arg at position 276, the much smaller neutral Thr occupies position 326 (*B. subtilis* numbering), avoiding unfavorable interactions.

in those enzymes in which position 276 is an Arg, position 326 is almost invariably a neutral residue (Thr, Ser, or Leu) or a glutamate, thus preventing electrostatic repulsion.

In comparing the molecular weights and K_d values for the wild-type and mutant enzymes, each mutant enzyme has a K_d value somewhat lower than that of the wild type; this result indicates that the equilibrium between the dimer and tetramer is shifted to favor the tetramer. It might be expected that since more tetramer is present at any given protein concentration, the mutants should have higher activity; however, if the tetramer is not assembled correctly, it may be less active.

Evidence for an incorrectly assembled tetramer is found in the thermal stability experiments. While the wild-type *B. subtilis* adenylosuccinate lyase is not completely stable at 42.5 °C over the time period studied, certain mutants are less stable. Substitutions of Ala, Ser, and Arg at position 276 resulted in enzymes that precipitated within the time period of the experiment. It is possible that areas of the enzyme usually buried are now exposed to solvent, resulting in incorrect subunit aggregation which is accelerated at the higher temperature of this experiment. It is interesting to note that the N276R/R326T enzyme is completely stable and does not exhibit the tendency to precipitate associated with the single mutant, N276R.

Further support for a role in subunit interactions, or the ability to interfere therein, for Asn^{276} is found in enzyme subunit complementation experiments. Intersubunit complementation is an event that arises from the formation of an active hybrid adenylosuccinate lyase enzyme from two different inactive enzymes (*29*−*31*). Complementation has provided functional evidence that residues from three subunits constitute the active site of adenylosuccinate lyase (*4*−*6*). This enzyme contains four active sites; theoretically, if there is complete randomization of subunits, 25% of the wild-type activity could be restored (*4*). We have previously shown that E275Q and H141Q adenylosuccinate lyases exhibit significantly less (6%) reactivation than the amount theoretically possible (*5*). Here, a mutation at position 276 (N276E) exhibits even less complementation (1%) with H141Q (data not shown). This subunit pair has a small locus of interaction (Figure 8C of ref *5*), and thus a structurally impaired mutant would only serve to magnify difficulties assembling into a properly formed tetramer, resulting in the drastically reduced levels of complementation that we have observed. In contrast, mixing N276E with the H68Q enzyme under optimal conditions yields up to 13% of wild-type activity, consistent with the larger area of interaction between the subunits contributing these two residues (Figure 8B of ref *5*).

The extent to which protein structure is altered in the mutant enzymes appears to be limited to the quaternary level. Figure 4 shows the CD spectra of the enzymes at higher concentration where they are all predominantly in the tetrameric state. The spectra of all the mutant and wild-type enzymes are virtually superimposable, indicating that the amount and type of secondary structure are not altered as a result of the mutations. Therefore, it seems that the differences observed between the wild-type and Asn^{276} mutant enzymes arise from altered subunit interactions.

The changes in subunit interactions may account for the altered catalytic properties in comparing the wild-type and mutant adenylosuccinate lyases. The lowered V_{max} values likely are a secondary effect of a nonoptimally assembled active site, as the most unstable enzymes are associated with the lowest V_{max} values. Likewise, the shifts in pK_2 could reflect slightly different positioning of ionizable groups in the active site (*6*).

For the mutant enzymes, their ability to handle either substrate was reduced relative to the wild-type adenylosuccinate. We have expressed the ratio of activity as SAICAR/SAMP to give comparable numbers to those reported in the literature, where the ratio is expressed in terms of the concentrations of dephosphorylated derivatives (i.e., S-Ado/SAICA riboside). If the activity toward SAICAR is higher, the concentration of succinyladenosine accumulating will be higher. The wild-type enzyme has a SAICAR/adenylosuccinate activity ratio of approximately 1.3. This ratio signifi-

cantly increases in those enzymes that are the most unstable and presumably have the greatest extent of structural abnormality. Enzymes such as N276R and N276E, which have relatively low activity toward adenylosuccinate, have respectively two to three times greater activity toward SAICAR. This difference in activity may be attributed to the relative flexibility of the substrates and the ability of a structurally impaired enzyme active site to accommodate them. SAICAR is an inherently more flexible molecule because it does not have the fused double ring of the purine adenylosuccinate.

Through a series of mutations at position 276 of adenylosuccinate lyase, we have elucidated the underlying biochemistry associated with one form of the human disease, adenylosuccinate lyase deficiency. The enzyme impairment resulting from mutations at this position, which gives rise to a mild variant of the disease in humans, appears to be predominantly structural in nature, rather than catalytic, as was previously speculated. The amino acid residue at position 276 appears to be important for proper subunit interaction and correct tetramer assembly.

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REFERENCES

1. Ratner, S. (1972) in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. 7, pp 167−197, Academic Press, New York.
2. Lee, T. T., Worby, C., Dixon, J. E., and Colman, R. F. (1997) *J. Biol. Chem. 272*, 458−465.
3. Lee, T. T., Worby, C., Bao, Z. Q., Dixon, J. E., and Colman, R. F. (1998) *Biochemistry 37*, 8481−8489.
4. Lee, T. T., Worby, C., Bao, Z. Q., Dixon, J. E., and Colman, R. F. (1999) *Biochemistry 38*, 22−32.
5. Brosius, J. L., and Colman, R. F. (2002) *Biochemistry 41*, 2217−2226.
6. Brosius, J. L., and Colman, R. F. (2000) *Biochemistry 39*, 13336−13343.
7. Toth, E. A., and Yeates, T. O. (2000) *Structure 8*, 163−174.
8. Weaver, T. M., Levitt, D. G., Donnelly, M. I., Wilkens Stevens, P. P., and Banaszak, L. J. (1995) *Nat. Struct. Biol. 2*, 654−662.
9. Simpson, A., Bateman, O., Driessen, H., Lindley, P., Moss, D., Mylvaganam, S., Narbor, E., and Slingsby, C. (1994) *Nat. Struct. Biol. 1*, 724−733.
10. Shi, W., Dunbar, J., Jayasekera, M. M. K., Viola, R. E., and Farber, G. K. (1997) *Biochemistry 36*, 9136−9144.
11. Turner, M. A., Simpson, A., McInnes, R. R., and Howell, L. P. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 9063−9068.
12. Jaeken, J., and Van den Berghe, G. (1984) *Lancet 2*, 1058−1061.
13. Adenylosuccinate Lyase Mutations Database Home Page (http://www.icp.ucl.ac.be/adsldb/mutations.html).
14. Jaeken, J., Wadman, S. K., Duran, M., van Sprang, F. J., Beemer, F. A., Holl, R. A., Theunissen, P. M., de Cock, P., Van den Berghe, F., Vincent, M.-F., and Van den Berghe, G. (1988) *Eur. J. Pediatr. 148*, 125−131.
15. Stone, T. W., Roberts, L. A., Morris, B. J., Jones, P. A., Ogilvy, H. A., Behan, W. M. H., Duley, J. A., Simmonds, H. A., Vincent, M. F., and van den Berghe, G. (1998) *Adv. Exp. Med. Biol. 431*, 185−189.
16. Ciardo, F., Salerno, C., and Curatolo, P. (2001) *J. Child Neurol. 16*, 301−308.
17. Race, V., Marie, S., Vincent, M.-F., and Van den Berghe, G. (2000) *Hum. Mol. Genet. 9*, 2159−2165.
18. Stone, R. L., Zalkin, H., and Dixon, J. E. (1993) *J. Biol. Chem. 286*, 19710−19716.
19. Stone, R. L., Aimi, J., Barshop, B. A., Jaeken, J., Van den Berghe, G., Zalkin, H., and Dixon, J. E. (1992) *Nat. Genet. 1*, 59−63.
20. Crocco, J. M., Brosius, J. L., and Colman, R. F. (2001) *FASEB J. 15*, A187 (Part 1).
21. Redinbo, M. R., Eide, S. M., Stone, R. L., Dixon, J. E., and Yeates, T. O. (1996) *Protein Sci. 5*, 786−788.
22. Laemmli, U. K. (1970) *Nature 227*, 680−685.
23. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
24. Tornheim, K., and Lowenstein, J. M. (1972) *J. Biol. Chem. 247*, 162−169.
25. Woodward, D. O., and Braymer, H. D. (1966) *J. Biol. Chem. 241*, 580−586.
26. Wyatt, P. J. (1993) *Anal. Chim. Acta 272*, 1−40.
27. Marie, S., Cuppens, H., Heuterspreute, M., Jaspers, M., Tola, E. Z., Gu, X. X., Legius, E., Vincent, M.-F., Jaeken, J., Cassiman, J.-J., and Van den Berghe, G. (1999) *Hum. Mutat. 13*, 197−202.
28. Yamaguchi, T., and Adachi, K. (2002) *Biochem. Biophys. Res. Commun. 290*, 1382−1387.
29. Woodward, D. O., Partridge, C. W. H., and Giles, N. H. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 1237−1244.
30. Woodward, D. O. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 846−850.
31. Woodward, D. O. (1960) *Q. Rev. Biol. 35*, 313−323.

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