A Key Role in Catalysis for His^{89} of Adenylosuccinate Lyase of *Bacillus subtilis*†

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ABSTRACT: Adenylosuccinate lyase of *Bacillus subtilis* is a tetrameric enzyme which catalyzes the cleavage of adenylosuccinate to AMP and fumarate. We have mutated His^{89}, one of three conserved histidines, to Gln, Ala, Glu, and Arg. The enzymes were expressed in *Escherichia coli* and purified to homogeneity. As compared to a specific activity of 1.56 μmol of adenylosuccinate converted/min/mg protein for wild-type enzyme, the mutant enzymes exhibit specific activities of 0.0225, 0.0036, 0.0036, and 0.0009 for H89Q, H89A, H89E, and H89R, respectively. Circular dichroism and FPLC gel filtration reveal that mutant enzymes have a similar conformation and oligomeric state to that of wild-type enzyme. In H89Q, the K_M for adenylosuccinate increases slightly to 2.5-fold that of wild-type, the K_M for fumarate is elevated 3.3-fold, and the K_M for AMP is 13 times higher than that observed in wild-type enzyme. The catalytic efficiency of the H89Q enzyme is compromised, with k_{cat}/K_M reduced 174-fold in the direction of AMP formation. These data suggest that His^{89} plays a role in both the binding of the AMP portion of the substrate and in correctly orienting the substrate for catalysis. Incubation of H89Q with inactive H141Q enzyme [Lee, T. T., Worby, C., Bao, Z.-Q., Dixon, J. E., and Colman, R. F. (1999) *Biochemistry 38*, 22−32] leads to a 30-fold increase in activity. This intersubunit complementation indicates that His^{89} and His^{141} from different subunits participate in the active site and that both are required for catalysis.

Adenylosuccinate lyase is an enzyme that intervenes twice in purine biosynthesis, producing precursors for both DNA and RNA synthesis (*1*). The first reaction catalyzed is the cleavage of 5-aminoimidazole-4-(*N*-succinylocarboxamide) ribonucleotide (SAICAR) to produce 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and fumarate. Later in the pathway, adenylosuccinate lyase aids in the conversion of IMP to AMP through the cleavage of adenylosuccinate to AMP and fumarate. This enzyme is also one of three that constitute the purine nucleotide cycle, providing fumarate to replenish the citric acid cycle.

Adenylosuccinate lyase has been implicated in several disease states in humans. Phenotypically, those with an adenylosuccinate lyase deficiency are characterized by mild to severe mental retardation, epilepsy, autistic features, and muscle wasting (*2*). To date, 16 point mutations and one splicing error have been identified (*3*). Many of these point mutations do not appear to be located in the active site and are thought to render the enzyme structurally unstable, yet catalytically competent.

Early kinetic studies showed that the reactions catalyzed by adenylosuccinate lyase proceed through a uni−bi mechanism, with a sequential release of product in which fumarate leaves the enzyme before AMP (*4*−*6*). The mechanism itself is considered to be a β-elimination (*7*). Such a reaction involves the attack of a general base on the β-H of adenylosuccinate, and the protonation of the N-1 ring nitrogen or the N-6 amino group by a general acid to facilitate the leaving of AMP. Previous affinity labeling and mutagenesis studies of *Bacillis subtilis* adenylosuccinate lyase have identified His^{68} and His^{141} as the residues functioning as the general acid and base, respectively (*8*−*10*). No other key residues in the active site have been identified.

Adenylosuccinate lyase is a member of the fumarase superfamily of metabolic enzymes, all of which catalyze reactions with fumarate as one of the products. Every member of the superfamily is a homotetramer with a molecular weight of approximately 200 kDa. Included in this family are aspartase, class II fumarase, δ-crystallin, argininosuccinate lyase, and adenylosuccinate lyase. These proteins all have highly conserved tertiary structures, yet relatively low sequence homology. Upon the basis of this high structural similarity, Lee et al. (*9*). constructed a homology model of *B. subtilis* adenylosuccinate lyase using the Modeler Program of Insight II (Figure 1A).

The crystal structure of the bacterial adenylosuccinate lyase from *Thermotoga maritima* has recently been published (*11*) and is shown in Figure 1B. The adenylosuccinate lyases from *B. subtilis* and *T. maritima* share 50% identity and an additional 23% strong similarity in amino acid sequence. Therefore, the crystal structure of *T. maritima* should provide an excellent guide to the structure of the enzyme from *B. subtilis*. As indicated in Figure 1, our homology model and the crystal structure are strikingly similar,[1] supporting the use of homology models as a guide to the selection of

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[1] The greatest discrepancy between the model and the crystal structure occurs in the region of the C-terminus where the sequence alignment between adenylosuccinate lyase and the protein standards used to construct the model was most divergent (*9*).

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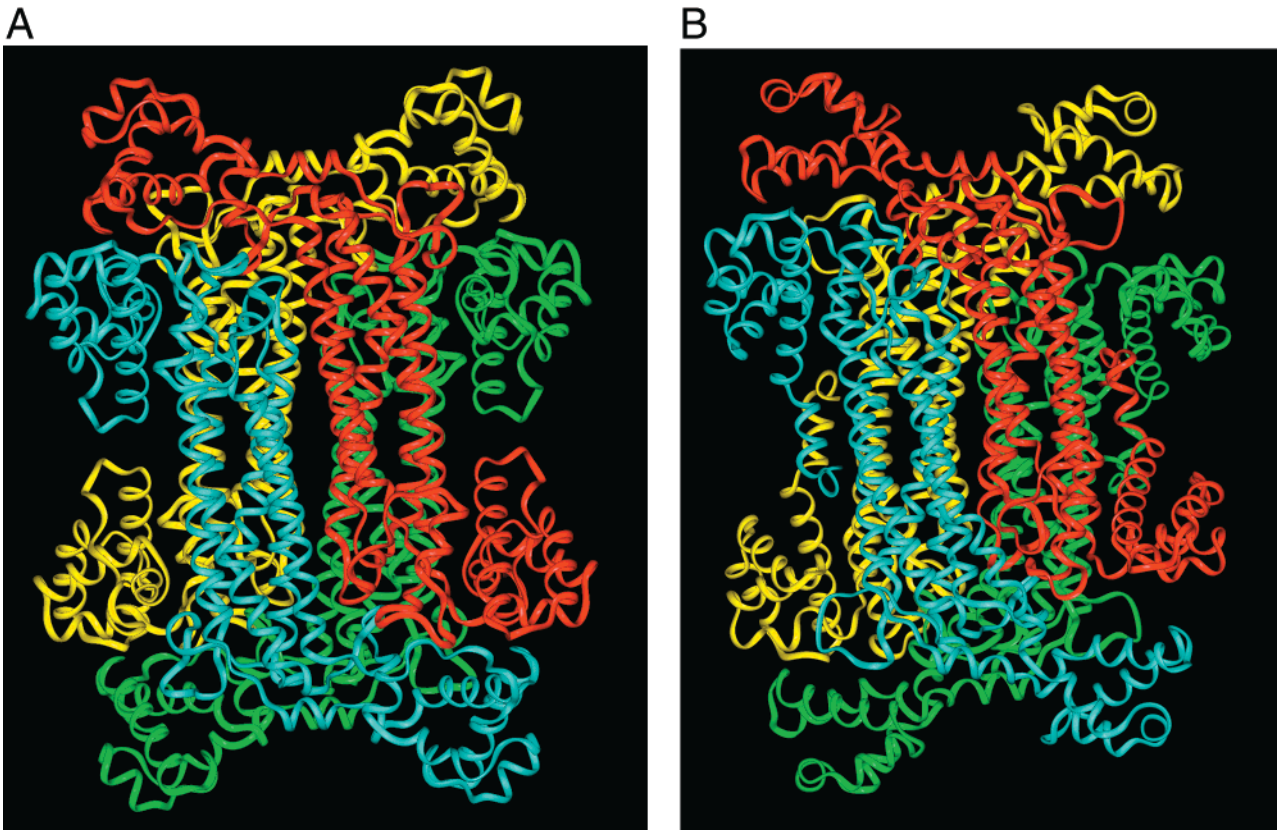

FIGURE 1: (A) Homology model of *Bacillis subtilis* adenylosuccinate based upon the crystal structures of *E. coli* fumarase, *E. coli* aspartase, human argininosuccinate lyase, and duck δ2-crystallin (*9*). (B) The crystal structure of adenylosuccinate lyse of *Thermotoga maritima* (*11*).

mutagenesis candidates.

Adenylosuccinate lyase from *B. subtilis* contains 11 histidines, three of which are completely conserved among the enzymes that have been sequenced from 28 species. Two of these histidines have previously been characterized: His[68] and His[141]. His[89] is the third conserved histidine. Upon the basis of its conservation and its proximity to the active-site region previously identified (*9*, *11*), His[89] was selected as a residue to study by site-directed mutagenesis.

This paper describes an evaluation by site-directed mutagenesis of the importance of His[89] of adenylosuccinate lyase. A preliminary version of this work has been presented (*12*).

EXPERIMENTAL PROCEDURES

Materials. Adenylosuccinate, adenosine 5′ monophosphate, fumarate, imidazole, MES,[2] HEPES, and TAPS were purchased from Sigma. Protein assay concentrate was obtained from Bio-Rad. The fluorescent sequencing primers were from LiCOR and the oligonucleotides were from Operon. All other chemicals were of reagent grade.

Site-Directed Mutagenesis. Mutations of pBHis were carried out using the QuikChange mutagenesis kit (Stratagene, LaJolla, CA). The following oligonucleotides and their complements were used to generate the His[89] mutations: GAA AGA AAA TGG GTG GAA TAC GGC TTA ACG (H89E), GAA AGA AAA TGG GTG CGT TAC GGC TTA ACG (H89R), GAA AGA AAA TGG GTG GCT TAC GGC TTA ACG (H89A), AA AGA AAA TGG GTG CAA TAC GGC TTA ACG T (H89Q). All mutations were confirmed by nucleotide sequencing by the Biology Core Facility, University of Delaware.

The pBHis plasmid, encoding adenylosuccinate lyase of *Bacillus subtilis*, was a generous gift from Dr. Jack E. Dixon (University of Michigan). pBHis was expressed in *E. coli* strain BL21(DE3) and the mutant enzymes purified to homogeneity, as previously described (*8*, *13*). The protein concentration was measured from its absorbance at 280 nm using $E^{1\%}_{280nm} = 10.6$ (*8*), except where noted. Both N-terminal amino acid gas-phase sequencing and electrophoresis in 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (*9*, *14*) were used to assess the purity of the enzyme. Protein sequencing was performed on an Applied Biosystems sequence analyzer, model 470A, with an on-line PTH analyzer, model 120 and computer model 900A. Approximately 500 pmol of purified protein was used.

Fast-Protein Liquid Chromatography of Wild-Type and Mutant Adenylosuccinate Lyase. The molecular weights and multimeric states of both mutant and wild-type adenylosuccinate lyase were determined by gel filtration. A Pharmacia Superose 12 (1 × 30 cm) column was used on a Pharmacia FPLC. The system was equilibrated in 20 mM potassium phosphate and 20 mM NaCl, pH 7.0, which maintains the enzyme in its native state. Protein samples, ranging from 0.12 to 0.50 mg/mL were loaded via a 200 μL injection loop with a flow rate of 0.3 mL/min. Molecular weight standards from Pharmacia Biotech were used to determine the size of the adenylosuccinate lyase samples.

[2] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TAPS, (*N*-tris)-[hydroxymethyl]methyl-3-aminopropane sulfonic acid.

Circular Dichroism of Adenylosuccinate Lyase. Circular dichroism experiments were carried out on a Jasco J-710 spectropolarimeter. Ellipticity as a function of wavelength was measured from 250 to 200 nm, in 0.1 nm increments, using a 0.1 cm cylindrical quartz cuvette. Enzyme samples (0.10−0.15 mg/mL) were dialyzed against 20 mM potassium phosphate containing 20 mM KCl (pH 7.0) and preincubated at 25 °C for 30 min prior to use. Final protein concentrations were determined using the Bio-Rad protein assay, based on the method of Bradford (*15*). The samples were scanned five times, averaged, and corrected for the background using buffer alone. The mean molar ellipticity $[\theta]$ (deg cm^2 dmol^{-1}) was calculated from the equation $[\theta] = \theta/(10nCl)$, 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 (437 for adenylosuccinate lyase, including the His_6 tag). [This is the same equation actually used in the earlier paper from this laboratory (*9*), although the equation printed in that paper contained a typographical error of 10-fold.]

Adenylosuccinate lyases (both wild-type and H89Q) in the absence of ligands were measured from pH 6.0 to 9.0, to test the possibility of conformational change. Potassium phosphate (20 mM), containing 20 mM KCl was used over the entire pH range. No change in conformation was observed.

Kinetics of B. subtilis Adenylosuccinate Lyases. The activities of mutant and wild-type enzymes were assayed from the time-dependent decrease in absorbance at 282 nm, using a difference extinction coefficient of 10 000 M^{-1} cm^{-1} between adenylosuccinate and AMP (*16*). Prior to assaying, the enzyme was incubated for 30 min at 25 °C in 20 mM potassium phosphate, pH 7.0, with 20 mM NaCl present. Assays under standard conditions were conducted in 50 mM HEPES, pH 7.0, at 25 °C. Unless otherwise noted, 60 μM adenylosuccinate was present in the assays. Specific activity, in the direction of AMP formation, is defined as micromoles of adenylosuccinate converted per minute per milligrams of enzyme.

The k_{cat} and K_M values were determined by varying the concentration of substrate present. The data were analyzed by Lineweaver−Burk plots, and error estimates were obtained from Sigma Plot software. In the reverse direction, 10 mM fumarate was present when the AMP concentration was varied, and 1 mM AMP was present when the fumarate concentration was varied. Due to high absorbance limitations at 280 nm, the rate in the reverse direction was monitored at 290 nm, using the difference extinction coefficient of 4050 M^{-1} cm^{-1}.

pH Profiles of Wild-Type and H89Q Adenylosuccinate Lyase. The pH dependence of V_{max} was determined for wild-type and H89Q enzymes from pH 6.0 to pH 8.5 and 8.2, respectively, at 25 °C. The buffers used were MES (pH 6.0−6.9), HEPES (pH 6.8−8.0), and TAPS (pH 7.9−8.5). For wild-type and H89Q, 60 and 100 μM adenylosuccinate, respectively, were present in the assay. A constant ionic strength of 0.03 M was maintained by adjusting the buffer concentration at each pH. The pH of each assay solution was measured after the rate determination.

Temperature Dependence of the pH Profiles of Wild-Type Adenylosuccinate Lyase. Possible shifts in the pH profile due to temperature were investigated for wild-type enzyme from pH 6.0 to 9.0, at 10, 20, and 30 °C. MES, HEPES, and TAPS, maintained at a constant ionic strength of 0.03 M, were used as buffers. Wild-type enzyme, 0.6 mg/mL, was preincubated at 25 °C for 30 min and maintained at this temperature for the duration of the experiment, as was the substrate (adenylosuccinate, 60 μM). Both the buffers and the spectrophotometer cell holder were maintained at the temperature being studied. The reaction was initiated by the addition of 20 μL of enzyme (0.6 mg/mL) to the buffer plus substrate. To determine the pH of the assay solution after the rate measurement, the pH meter was calibrated with 0.05 M phosphate buffer, pH 7.0 (Fisher Scientific), and the pH of each assay solution was determined at the given temperature.

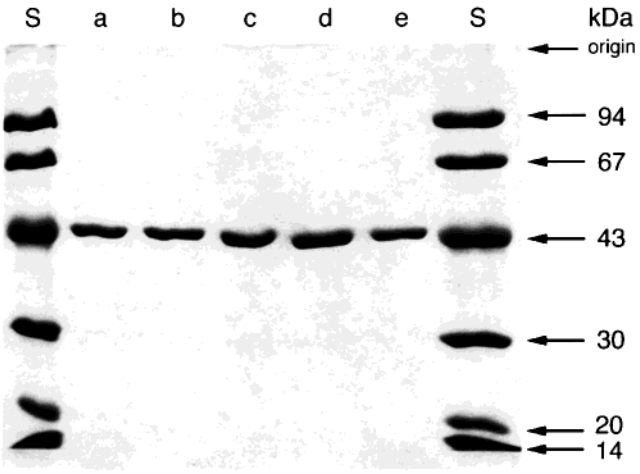

FIGURE 2: Electrophoresis in 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. Protein samples were: (S) protein standards, (a) wild-type, (b) H89Q, (c) H89E, (d) H89A, (e) H89R enzymes. The amount of adenylosuccinate lyase in every lane was 2.4 μg.

Intersubunit Complementation of Mutant Enzymes. Combinations of H141Q, H68Q (*9*) and H89Q adenylosuccinate lyases were tested for intersubunit complementation. Each mutant (1.4 mg/mL) was incubated separately for 1.5 h at 25 °C in 20 mM potassium phosphate with 20 mM NaCl present (pH 7.0). Equal volumes were mixed, with the final concentration of each mutant being 0.7 mg/mL. Reactivation was followed by assaying over time. Wild-type enzyme (1.4 mg/mL) served as a control.

RESULTS

Activity and Purity of His^{89} Adenylosuccinate Lyase Mutants. Glutamine, glutamate, alanine, and arginine mutants of His^{89}, one of three completely conserved histidines in adenylosuccinate lyase, were constructed, expressed, and purified. The resulting enzymes were homogeneous, as shown by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (Figure 2). Furthermore, N-terminal amino acid sequencing verified that there was no contamination from the *E. coli* enzyme, as the two proteins differ in their first 16 residues. The specific activity of each mutant, defined as micromoles of substrate converted per minute per milligram of protein, was evaluated in the direction of AMP formation, as summarized in Table 1. All mutants show a marked decrease in specific activity compared to wild-type enzyme, suggesting an important role for His^{89}.

Gel Filtration FPLC of Wild-Type and Mutant Adenylosuccinate Lyase. In its active form, adenylosuccinate lyase

Table 1: Specific Activity for Wild-Type and His[89] Mutants of Adenylosuccinate Lyase

enzyme	specific activity[a] (μmol/min/mg)
wild-type	1.5600
H89Q	0.0225
H89E	0.0036
H89A	0.0036
H89R	0.0009

[a] The specific activities were measured at 25 °C in 50 mM. HEPES buffer, pH 7.0, containing 60 μM adenylosuccinate.

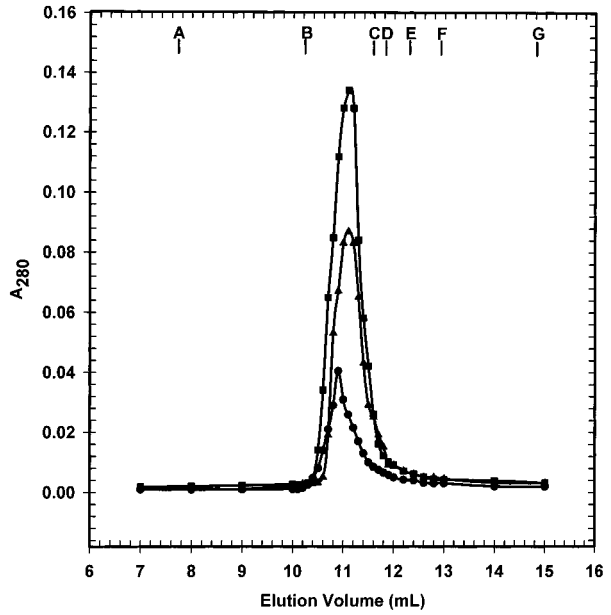

FIGURE 3: Elution profile of wild-type and mutant adenylosuccinate lyase from a Superose-12 FPLC column. The multimeric states of the adenylosuccinate lyases were determined using the following protein standards: ferritin (B, 440 kDa), catalase (C, 232 kDa), aldolase (D, 158 kDa), bovine serum albumin (E, 67 kDa), ovalbumin (F, 43 kDa), and chymotrypsinogen (G, 25 kDa). The total volume (V_t) of the column is 25 mL, and the void volume (V_o) as determined by Blue Dextran (A) is 8.0 mL. (■) Wild-type, (▲) H89Q, (●) H89A. V_e is taken as the volume of the elution peak of a given sample.

and other members of the superfamily exist as homotetramers with molecular weights of approximately 200 kDa (*9*). To evaluate whether the mutations caused disruption of the tetrameric state, gel filtration FPLC was conducted at room temperature under nondenaturing conditions in 20 mM potassium phosphate, pH 7.0, containing 20 mM KCl (Figure 3). To determine the molecular weight of adenylosuccinate lyase, and hence its oligomeric state, $(V_e - V_o)/(V_t - V_o)$ vs $\log(M_r)$ was plotted for the protein standards. Wild-type enzyme eluted between ferritin (B) and catalase (C), with an estimated molecular weight of 245 kDa. No absorbance was seen in either the monomer or dimer region. These results are consistent with adenylosuccinate lyase existing as a tetramer.

Figure 3 also shows the data for the H89Q and H89A mutants, as representative; these, as well as H89E, eluted at about the same position as wild-type enzyme, indicating that the mutations do not prevent tetramer formation. However, no peaks on the FPLC were observed for H89R. It appears that this mutant is unstable during gel filtration, perhaps indicating the severity of the mutation.

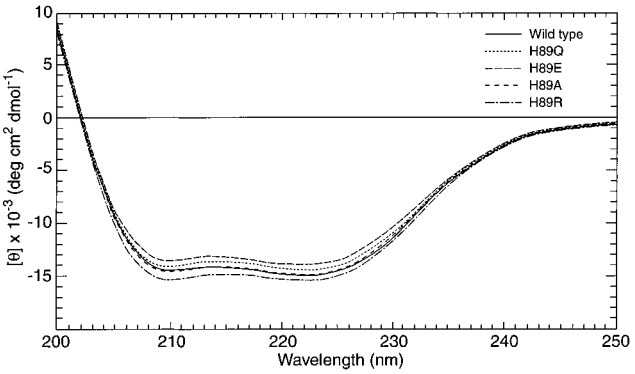

FIGURE 4: Circular dichroism spectra of wild-type and mutant enzymes in the far-UV region. Each spectrum has been corrected for the background using buffer alone, and has been normalized to the same molar concentration. Minima occur at 208 and 222 nm.

Circular Dichroism Spectroscopy of Wild-Type and Mutant Enzymes. To determine if the reduced activity of the mutants results from conformational changes, circular dichroism was utilized. The H89Q, H89A, H89E, and H89R spectra (Figure 4) have shapes and intensities similar to that of wild-type enzyme, indicating that the mutations do not cause major changes in secondary structure. The typical α-helix features dominate, with minima occurring at 208 and 222 nm, consistent with the high content of α-helix found in members of the superfamily (*9*).

Kinetic Parameters of Wild-Type and H89Q Enzyme. The kinetic parameters of adenylosuccinate lyase were measured both in the direction of AMP formation ("forward reaction") and in the direction of adenylosuccinate formation ("reverse reaction"). H89Q was the only mutant with sufficient activity to examine its kinetic properties in further detail.

Table 2 summarizes the kinetic data for wild-type and H89Q enzyme in the forward direction. The K_M for adenylosuccinate in the mutant is only slightly higher than that of wild-type. More dramatic are the reductions in V_{max} (and k_{cat}): both parameters are 70 times higher in wild-type than in the H89Q mutant. The catalytic efficiency (k_{cat}/K_M) of wild-type is 174 times that of the H89Q mutant enzyme.

Table 3 summarizes the kinetic parameters in the direction of adenylosuccinate formation. In this direction, both K_M values and k_{cat} are altered due to the mutation. The K_M for AMP is 13 times higher in H89Q than in wild-type enzyme, while the K_M for fumarate is only slightly elevated. The decreases in k_{cat} and V_{max} in the mutant are also significant, each being 3.4 times higher in wild-type than in H89Q enzyme. These results imply a role for His[89] that appears to involve both catalysis and binding in the direction of adenylosuccinate formation, and catalysis in the direction of AMP formation.

pH Dependence of the Reaction Catalyzed for both Wild-Type and H89Q Enzyme. To evaluate the impact of the H89Q mutation upon the ionizable groups in the enzyme which influence activity, the kinetics were studied at various pHs in the forward direction. Throughout the range studied, from pH 6.0 to 8.5, the K_M values changed relatively little and are less than 2-fold higher in H89Q as compared to wild-type (Table 4). At each pH, the velocities measured at the highest concentration of adenylosuccinate used were extrapolated to V_{max} using the K_M values, and are expressed in terms of specific activity, as shown in Figure 5.

Table 2: Kinetic Parameters of Wild-Type and H89Q Mutant Adenylosuccinate Lyase in the Direction of AMP Formation

enzyme sample	V_{max} (μmol/min/mg)	K_M (μM) (adenylosuccinate)	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
H89Q	0.022 ± 0.0009	8.66 ± 0.38	0.0185 ± 0.0007	0.0213×10^5

Table 3: Kinetic Parameters of Wild-Type and His[89] Mutant Adenylosuccinate Lyase in the Direction of Adenylosuccinate Formation

enzyme sample	V_{max} (μmol/min/mg)	k_{cat} (s^{-1})	K_m (mM) (AMP)	K_m (mM) (fumarate)
wild-type	3.27 ± 0.14	2.69 ± 0.11	0.06 ± 0.005	1.54 ± 0.09
H89Q	0.95 ± 0.09	0.80 ± 0.07	0.79 ± 0.09	5.07 ± 0.67

Table 4: pH Dependence of K_M for Adenylosuccinate of Wild-Type and H89Q Enzyme

pH	K_M (μM wild-type)	K_M (μM H89Q)
6.5	8.6	15.7
7.0	3.5	8.66
7.5	12.8	11.7
8.2	32.4	24.6
8.5	37.8	67.7

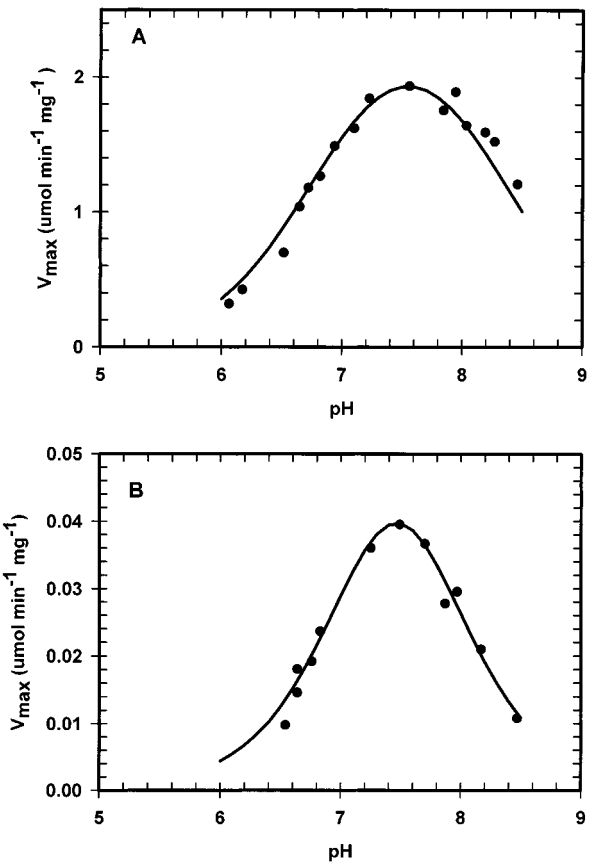

FIGURE 5: pH profiles for both wild-type and H89Q enzyme. The data have been extrapolated to V_{max} and expressed as specific activity. (A) Wild-type. (B) H89Q mutant enzyme. pK_1 and pK_2 were determined to be 6.95 and 8.60 for wild-type enzyme, and 7.3 and 7.6 for H89Q, respectively.

Both wild-type and H89Q yielded bell-shaped curves. Because the p*K*s are within 3 pH units of each other, initial values were estimated via the method of Alberty and Massey (*17*). The data were then fit to the following equation (*18*),

$$V_{max} = V_o/(1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)})$$

where V_{max} is the maximum velocity at a given pH, V_o is the intrinsic, pH independent value of V, and pK_1 and pK_2 are the pK values obtained for the left and right sides of the profile. SigmaPlot was used to analyze the data. Both profiles reflect extrapolated V_{max} values and are shown in Figure 5, with different velocity scales, to emphasize the much lower activity of the H89Q enzyme as compared with that of wild-type. Values of 6.95 and 8.60 were obtained for pK_1 and pK_2, respectively, in wild-type adenylosuccinate lyase. (These values differ from those previously published, as different buffers and salt concentrations were used).[3] In the H89Q mutant, pK_1 is increased to 7.3, while pK_2 is decreased to 7.6 relative to wild-type enzyme. As the greatest effect of the mutation upon the pH profile is on the right limb, it appears that His[89] may play a role in general acid catalysis.

Temperature-Dependent pH Profiles of Wild-Type Adenylosuccinate Lyase. In an attempt to identify the ionizable groups reflected in the wild-type pH rate profile, the pH dependence of the profile was examined from 10 to 30 °C. The K_M values for adenylosuccinate did not change significantly from their values at 25 °C (data not shown). The data were analyzed by the method described for the profile at 25 °C (Figure 6). Table 5 summarizes the p*K* values obtained over the temperature range; it is apparent that the pK_1 values do not change with temperature, while the pK_2 values decrease with increasing temperature. From the p*K* values, a plot of $1/T$ vs p*K* was generated to determine a ΔH_i value for each limb of the pH profile. A ΔH_i value of approximately 0 kcal/mol was obtained for the left limb, and a ΔH_i value of 9.9 kcal/mol was determined for the right limb of the profile. These surprising results imply that a histidine is not the ionizable group reflected in pK_1.

Intersubunit Complementation of His[89] and His[141] Mutants. Intersubunit complementation is a phenomenon manifested by some multimeric proteins, highly suggestive that the active sites are shared between subunits. Previous work in our laboratory has shown that adenylosuccinate lyase exhibits intersubunit complementation in vitro (*9*). If inactive enzymes with mutations at different residues are combined, each different subunit can contribute a wild-type residue to create a wild-type like active site and thereby restore activity. From complementation studies it is also possible to determine which subunits contribute to the active site.

His[89] and His[141] mutants are complementary, while His[89] and His[68] mutants are not (Figure 7). Each mutant was incubated separately for 1 h at 25 °C and then combined. Upon mixing, reactivation (if present) was seen almost immediately and reached a maximum point within approximately 2 h. A 30-fold increase in activity was observed for H89Q and H141Q. Our previous work showed that

[3] The previously published values of pK_1 and pK_2 were 7.3 and 8.0, respectively, for wild-type adenylosuccinate lyase (*9*).

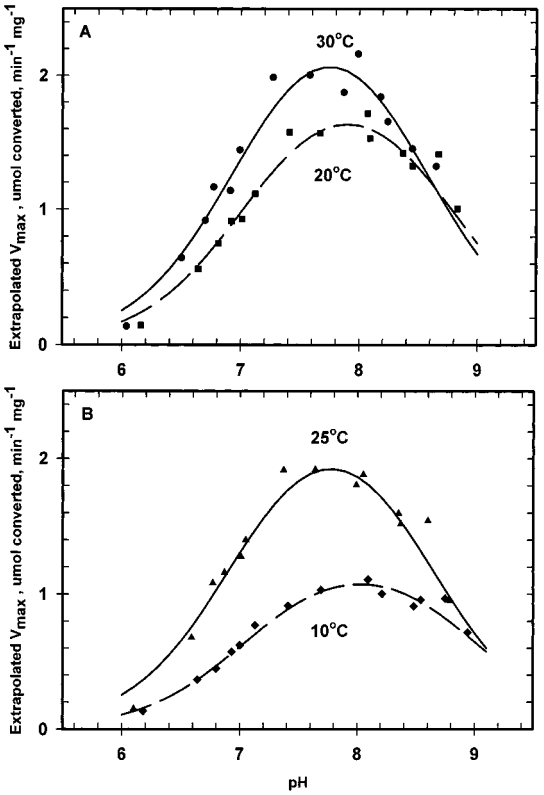

FIGURE 6: Temperature dependence of pH profiles for wild-type enzyme. (A) 30 °C (●) and 20 °C (■). (B) 25 °C (▲) and 10 °C (◆). The symbols represent experimental data points, while the curves are theoretical.

Table 5: p*K* Values Obtained from Temperature Dependent pH Rate Profiles of Wild-Type Adenylosuccinate Lyase

temperature (°C)	pK_1	pK_2
10.0	7.05	9.00
20.0	7.05	8.75
25.0	6.95	8.60
30.0	7.00	8.50

reactivation is not dependent upon the specific amino acid substitution; rather, the location of the residue is the key factor. Therefore, only the most active mutant, H89Q, was tested for complementation against either H141Q or H68Q.

A K_M of 0.24 mM for AMP was determined for the His[89]-His[141] hybrid. This value is only 4-fold higher than the wild-type value, suggesting that only minor distortion of the enzyme occurs as a result of either the mutations or hybrid formation. These results indicate that His[89] and His[141] are contributed to the active site from different subunits, while His[89] and His[68] come from the same subunit.

DISCUSSION

Characterization of the highly conserved His[89] by mutagenesis has revealed the importance of this residue. Substitution for His[89] of each of four amino acids leads to at least a 70-fold decrease in specific activity. The selections for replacement amino acids were made to test the importance of size, charge, hydrophobicity, and hydrogen bonding ability in the region of His[89]. Histidine was replaced with glutamine, a residue similar in size, hydrophobicity, and hydrogen-bonding potential; glutamine, however, cannot donate or accept a proton. Replacement by arginine was used to examine the effect of a positively charged residue that is only slightly larger than histidine. The substitution of glutamate allowed the assessment of a negatively charged residue at the position of His[89]. Finally, to further evaluate the importance of size and hydrophobicity, the smaller alanine was substituted for histidine. The strikingly reduced activity of all of these mutants reveals that not only the size of the residue, but also its charge, is important for activity. The most critical requirement for the amino acid at position 89 appears to be its ability to hydrogen bond, as H89Q was the most active mutant.

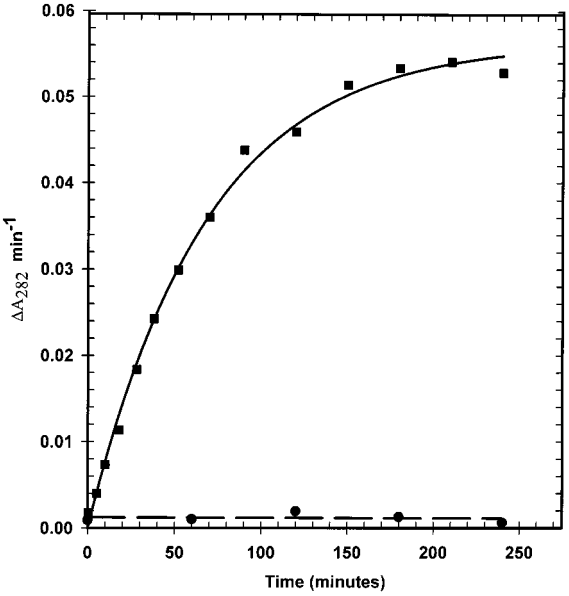

FIGURE 7: Complementation studies and reactivation of mutant enzymes. Each mutant was incubated separately, and then the two were mixed at time zero. The final concentration of each mutant in the mixture was 0.7 mg/mL. The specific activity of the hybrid after reactivation was compared to the specific activity of wild-type to determine that 21% of the activity was restored to the mutants. (■) H89Q + H141Q, (●) H89Q + H68Q.

Circular dichroism spectroscopy and gel filtration FPLC provide evidence that the reduced activity of the His[89] mutant enzymes is not due to appreciable conformational changes of the enzyme or alterations in its oligomeric state. From these results, it may be concluded that the replacement of His[89] does not cause major structural changes in adenylosuccinate lyase.

Examination of the kinetic parameters of mutant and wild-type enzymes offers insight into the role of His[89] in adenylosuccinate lyase. The K_M for adenylosuccinate only changes by a factor of 2.5 in H89Q compared to that of wild-type enzyme. However, in the reverse direction (adenylosuccinate formation), the K_M for fumarate is elevated 3.3-fold, and the K_M for AMP is 13 times higher than that of wild-type. These K_M results suggest that His[89] plays a major role in binding the AMP portion of substrate. It might have been expected that the K_M for adenylosuccinate would be increased more significantly, but, that was not observed. We interpret this result to indicate that the carboxylate groups of adenylosuccinate provide the major energy of interaction with the enzyme. In contrast, the predominant contributor to the much weaker binding interaction of AMP (which lacks the carboxylate groups) is probably the phosphate group

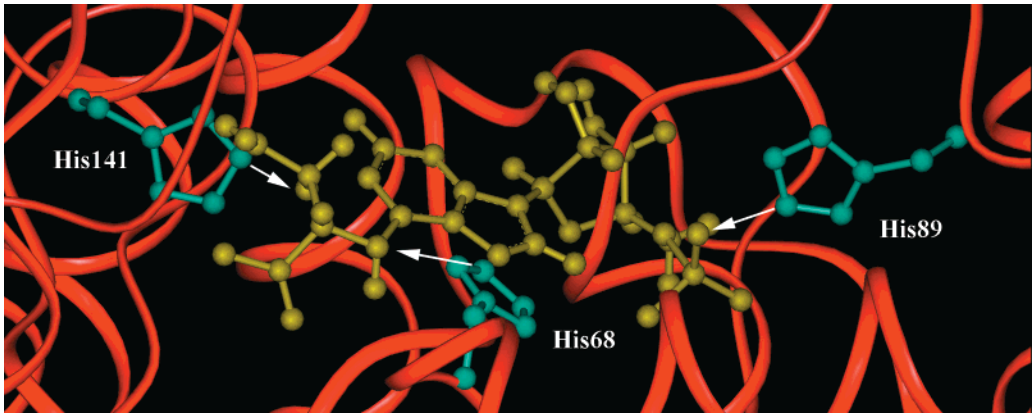

FIGURE 8: View of the postulated arrangement of substrate at the active site of adenylosuccinate lyase. Adenylosuccinate in its anti-conformation, was positioned into the crystal structure of adenylosuccinate lyase of *T. maritima*. Based on this placement, His^{141} and His^{68} are well positioned to function as the general base and general acid, respectively. His^{89} interacts closely with the phosphoryl group of adenylosuccinate.

which interacts with His^{89}. Thus, mutation of His^{89} has a greater effect on the affinity of the enzyme for AMP than for adenylosuccinate.

In both directions of catalysis, k_{cat}/K_M was decreased. In fact, in the direction of AMP formation, the effect of the mutation is only reflected in a change in k_{cat}, which was initially puzzling. However, proper orientation of the substrate is critical for catalysis: the interaction of His^{89} with the 5′-phosphate of the adenylosuccinate may be reflected in the 70-fold decrease in k_{cat} in H89Q in the direction of AMP formation, but be distributed between the effects on k_{cat} and K_Ms in the direction of adenylosuccinate formation. These results suggest that adenylosuccinate, despite the presence of the carboxylate groups, is even more difficult for H89Q to orient for catalysis than AMP and fumarate in the reverse direction. The difficulty in orientation may lie in the fact that the AMP portion of adenylosuccinate is constrained by the covalent linkage to the succinyl portion of the molecule, whereas the greater flexibility of free AMP and free fumarate may make them easier to align correctly for catalysis.

The pK_1 and pK_2 values for wild-type enzyme are 6.95 and 8.60, respectively. The values obtained for the H89Q mutant shifted to 7.3 for pK_1 and to 7.6 for pK_2. To aid in the assignment of these pK values to particular classes of ionizable groups, heats of ionization were determined for each limb of the pH rate profile for wild-type adenylosuccinate lyase. A value of approximately 0 kcal/mol was obtained for the left limb and 9.9 kcal/mol for the right limb. A ΔH_i value of 0 kcal/mol is characteristic of a neutral acid, such as a carboxyl group or phosphoric acid (*19*); thus, pK_1 cannot be identified with the ionization of His^{141}. It is likely that the pK of His^{141} is lower than pK_1 and that His^{141} is unprotonated over the entire range studied. Rather, pK_1 is probably due to the dissociation of a proton from the substrate's phosphoryl moiety or a carboxyl group in the enzyme−substrate complex. Further distinctions between these possibilities are currently under investigation in this laboratory.

The ΔH_i of 9.9 kcal/mol for the alkaline limb of the pH−V_{max} curve is likely associated with the ionization of a histidine. While a histidine typically has a ΔH_i value of 7 kcal/mol at pK 6.0, this value can be expected to increase by ~1.2 kcal/mol for each unit the pK is increased (*19*). Given that mutagenesis of two histidines has previously implicated them in catalysis (*9*) and a third important histidine has been identified in this paper, it is reasonable that the right limb of the pH profile represents at least one histidine. The roles of general base and general acid in the catalytic reaction were previously assigned to His^{141} and His^{68}, respectively (*9*). A significant decrease in pK_2 was observed in the H89Q enzyme. We propose that pK_2 reflects the ionization of two residues, both His^{68} and His^{89}; when His^{89} is changed by mutation to a nonionizable amino acid, the pH-profile solely reflects the ionization of His^{68}.

The recent publication of the crystal structure of adenylosuccinate lyase from *T. maritima* is consistent with our findings (*11*). As shown in Figure 8, the substrate, adenylosuccinate, was manually positioned in the crystal structure, on the basis of information provided by previous mutagenesis and affinity labeling studies (*8*−*10*). An anti-conformation was used for adenylosuccinate, since this is the most common conformation in which adenine nucleotides bind to enzymes (*20*, *21*).

Figure 8 provides valuable insights into the arrangement of the active site. His^{141} and His^{68} are positioned to serve as the general base and general acid, respectively. The ϵN of His^{141} is only 2.4 Å from the β-H of adenylosuccinate that is abstracted during the reaction. The ϵN of His^{68} is 2.9 Å from the N-6 amino group of substrate that is protonated during catalysis. More relevant to the present study, the ϵN of His^{89} is only 2.4 Å from the phosphoryl oxygen of adenylosuccinate in the anti-conformation. His^{89} is well positioned to interact with the phosphate group both to facilitate binding and to orient the substrate for catalysis. It is probable that one role of His^{89} is to contribute a hydrogen bond to the phosphoryl oxygen of substrate.

On the basis of the placement of the substrate and our experimental results, it seems likely that His^{89} exists in the protonated state in wild-type enzyme. Upon mutation of histidine to glutamine in this position, pK_2 decreases one logarithmic unit to 7.60. From this result, we can infer that His^{89} has a relatively high pK in wild-type enzyme, so that the residue would be protonated under physiological conditions. However, contrary to what might be expected, the positively charged H89R adenylosuccinate lyase was one of the least active mutant enzymes. On the basis of the crystal structure and the fact that arginine is a larger residue than

histidine, the size of the arginine may perturb the region so that efficient catalysis can no longer take place. This explanation can account not only for the low activity of this mutant, but also for its apparent structural instability (i.e., the precipitation problems associated with H89R).

The His[89] mutants also provide insight into the subunit contributors to the active site. Because the active site of adenylosuccinate lyase is constituted from amino acids of at least two subunits, complementation experiments may be utilized to elucidate which amino acids are contributed by each particular subunit (*22*−*25*). Briefly, the key residues from one subunit, e.g., His[141] and His[89], are physically too far apart to be located in the same active site; therefore more than one subunit must contribute amino acid side chains to form the active site. If the pair of inactive mutant enzymes are incubated, restoration of enzyme activity may be observed upon formation of wild-type like active sites. In order for this to occur, there must be an equilibrium in solution between the tetramer and its subunits. If it is assumed that there is random reassociation of monomers containing two different mutations (e.g., His[89] and His[141]), then 25% of wild-type activity could be restored (*9*). Experimentally, we observed 21% reactivation relative to wild-type by incubating H89Q and H141Q. This is slightly higher than the 15% reactivation we previously reported for His[68] and His[141] mutants, but still may reflect the fact that the scrambling of mutant subunits is not totally random (*9*). In contrast, no significant reactivation was observed upon incubation of H89Q and H68Q enzymes. On the basis of the crystal structure of the enzyme, this result accords with expectation: His[68] and His[89] are relatively close together (9 Å) on one subunit, while His[89] and His[141] are separated by almost 60 Å within one subunit. Therefore, it is reasonable that His[89] and His[68] are contributed to the active sites by one subunit, while His[89] and His[141] are contributed by two different subunits.

In conclusion, the results of this paper demonstrate that the conserved His[89] performs an important role in the reaction catalyzed by adenylosuccinate lyase. Since H89Q is the most active mutant, the normal role of His[89] may depend on its hydrogen-bonding ability, not only to enhance the binding of the substrate's phosphoryl group, but to correctly orient adenylosuccinate for catalysis.

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