# Three Subunits Contribute Amino Acids to the Active Site of Tetrameric Adenylosuccinate Lyase: $Lys^{268}$ and $Glu^{275}$ Are Required†

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ABSTRACT: Tetrameric adenylosuccinate lyase (ASL) of *Bacillus subtilis* catalyzes the cleavage of adenylosuccinate to form AMP and fumarate. We previously reported that two distinct subunits contribute residues to each active site, including the $His^{68}$ and $His^{89}$ from one and $His^{141}$ from a second subunit [Brosius, J. L., and Colman, R. F. (2000) *Biochemistry 39,* 13336−13343]. $Glu^{275}$ is 2.8 Å from $His^{141}$ in the ASL crystal structure, and $Lys^{268}$ is also in the active site region; $Glu^{275}$ and $Lys^{268}$ come from a third, distinct subunit. Using site-directed mutagenesis, we have replaced $Lys^{268}$ by Arg, Gln, Glu, and Ala, with specific activities of the purified mutant enzymes being 0.055, 0.00069, 0.00028, and 0.0, respectively, compared to 1.56 units/mg for wild-type (WT) enzyme. $Glu^{275}$ was substituted by Gln, Asp, Ala, and Arg; none of these homogeneous mutant enzymes has detectable activity. Circular dichroism and light scattering reveal that neither the secondary structure nor the oligomeric state of the $Lys^{268}$ mutant enzymes has been perturbed. Native gel electrophoresis and circular dichroism indicate that the $Glu^{275}$ mutant enzymes are tetramers, but their conformation is altered slightly. For K268R, the $K_m$s for all substrates are similar to WT enzyme. Binding studies using [2-$^3$H]-adenylosuccinate reveal that none of the $Glu^{275}$ mutant enzymes, nor inactive K268A, can bind substrate. We propose that $Lys^{268}$ participates in binding substrate and that $Glu^{275}$ is essential for catalysis because of its interaction with $His^{141}$. Incubation of H89Q with K268Q or E275Q leads to restoration of up to 16% WT activity, while incubation of H141Q with K268Q or E275Q results in 6% WT activity. These complementation studies provide the first functional evidence that a third subunit contributes residues to each intersubunit active site of ASL. Thus, adenylosuccinate lyase has four active sites per enzyme tetramer, each of which is formed from regions of three subunits.

Adenylosuccinate lyase is an enzyme that participates in the purine biosynthetic pathway, catalyzing the cleavage of adenylosuccinate to form AMP[1] and fumarate (*1*). This enzyme was associated with a human disease in 1984, making it of particular interest to study (*2*). Adenylosuccinate lyase deficiency arises from single point mutations to the gene, resulting in mild to severe mental retardation, epilepsy, muscle wasting, and autistic features. These mutations are located throughout the enzyme, with the majority being located relatively far from the active site, appearing to affect enzyme stability more than catalysis (*3*−*6*).

Kinetic studies have established that reaction occurs through a uni-bi mechanism, with fumarate leaving the enzyme before AMP (*7*−*9*). The overall reaction is a $\beta$-elimination in which the $\beta$-H of the succinyl moiety of adenylosuccinate is abstracted by a general base and the leaving group (AMP) is protonated at either the 1 or the 6 position by a general acid (*1, 10*). Through both affinity-labeling and site-directed mutagenesis studies of the *Bacillus subtilis* enzyme, $His^{68}$ and $His^{141}$ have been identified as the general acid and base (*11*−*13*). $His^{89}$ was shown to play a role in binding the AMP portion of substrate (*14*). Adenylosuccinate lyase exists as a homotetramer with a subunit molecular mass of 50 kDa. We have proposed that the enzyme has four active sites per tetramer and that each active site is formed by at least two distinct subunits, one containing $His^{141}$ and the other both $His^{68}$ and $His^{89}$ (*12, 14, 15*). The first crystal structure of an adenylosuccinate lyase (from *Thermotoga maritima*) was published in 2000 (*15*), and is consistent with four active sites.

Adenylosuccinate lyase is a member of a fumarase superfamily of enzymes, all of which share the same structural fold, yet have relatively low pairwise sequence identities. Members of this family include aspartase, class II fumarase, argininosuccinate lyase, $\delta$-crystallin, and adenylosuccinate lyase. In addition to their structural similarity, these enzymes all contain a highly conserved "signature sequence" region, shown in Figure 1. Of the adenylosuccinate lyases sequenced, 44 sources to date, the conserved region begins at $Gly^{261}$ and extends through $Glu^{275}$. The function of this region has not yet been investigated for adenylosuccinate lyase.

The crystal structure, shown in Figures 6 and 7, illustrates that the residues which are part of the "signature sequence" are in close proximity to the active site, and come from a

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CD, circular dichroism; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; AMP, adenosine 5′-monophosphate.

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| | Lys[268] | Glu[275] |
|---|---|---|
| *B. subtilis* | KGQK**GSS**A**M**PH**K**R**N**PIGS**E**NMTG |
| *T. maritima* | KGQR**GSS**A**M**PH**K**K**N**PITC**E**RLTG |
| *E. coli* | AGEI**GSS**T**M**PH**K**V**N**PIDF**E**NSEG |
| Human | KQQI**GSS**A**M**PY**K**R**N**PMRS**E**RCCS |
| Chicken | KDQI**GSS**A**M**PY**K**R**N**PMRS**E**RCCS |
| Yeast | KSQI**GSS**A**M**AY**K**R**N**PMRC**E**RVCS |

FIGURE 1: Sequence alignment of the "signature sequence" region of representative adenylosuccinate lyases. The residues in boldface font are conserved among all 44 adenylosuccinate lyases sequenced to date. (Pro[271] is conserved in most, but not all, ASLs.) Most of these residues, including Lys[268] and Glu[275], are also completely conserved across the superfamily. The numbering is that of the *B. subtilis* enzyme.

third subunit, which has not previously been implicated functionally as contributing to catalysis. Based on their conservation in the "signature sequence," and their physical proximity to other known active site residues, Lys[268] and Glu[275] were selected for study by site-directed mutagenesis. Furthermore, from the crystal structure, Glu[275] is positioned very close (2.8 Å) to the catalytic base, His[141]. We considered that a Glu[275]−His[141] interaction may be important for enzyme function. The aim of this project was to elucidate the catalytic role of each residue and to investigate the subunit interactions needed for proper enzyme function. Portions of this work have been presented in preliminary form (*16*).

## EXPERIMENTAL PROCEDURES

*Materials*. Adenylosuccinate, adenosine 5′-monophosphate, fumarate, HEPES,[1] and imidazole were purchased from Sigma. [2-$^3$H]-Adenosine 5′-monophosphate was from Amersham Pharmacia Biotech. Oligonucleotides for both sequencing and site-directed mutagenesis were obtained from Biosynthesis. The protein assay concentrate was from BioRad. All other chemicals were of reagent grade.

*Site-Directed Mutagenesis.* Mutations to the pBHis plasmid (a gift from Dr. Jack E. Dixon, University of Michigan), which encodes adenylosuccinate lyase of *Bacillus subtilis*, were constructed using the Stratagene QuikChange mutagenesis kit. The following oligonucleotides, and their complements, were used to generate the Glu[275] mutant enzymes: CCG ATT GGC TCT GCA AAC ATG ACA GGC (Ala), CCG ATT GGC TCT GAT AAC ATG ACA GGC (Asp), CCG ATT GGC TCT CAA AAC ATG ACA GGC (Gln), and CCG ATT GGC TCT AAA AAC ATG ACA GGC (Lys). The Lys[268] mutant enzymes were generated from the following oligonucleotides, and their complements: GCA ATG CCG CAT GCA CGA AAT CCG ATT GCG (Ala), GCA ATG CCG CAT AGA CGA AAT CCG ATT GCG (Arg), GCA ATG CCG CAT CAA CGA AAT CCG ATT GCG (Gln), and GCA ATG CCG CAT GAA CGA AAT CCG ATT GCG (Glu). The presence of the mutations was confirmed by DNA sequencing, which was carried out either at the Delaware Biotechnology Institute and University of Delaware Center for Agricultural Biotechnology using an ABI Prism model 377 DNA sequencer (PE Biosystems) or at the University of Delaware Cell Biology Core Facility using a Long Readir 4200 DNA sequencer (Li-Cor, Inc., Lincoln, NE).

The pBHis plasmid, which encodes a 6-His tag on the N-terminal of adenylosuccinate lyase, was expressed in *E. coli* strain BL21(DE3), and the enzymes were purified to homogeneity using a nickel resin as described previously (*11*, *17*). Except where noted, protein concentrations were determined by the absorbance at 280 nm using $E^{1\%}_{280nm} = 10.6$ (*1*). Polyacrylamide gels (12%) containing 0.1% sodium dodecyl sulfate were used to evaluate the purity of the resultant enzymes (*18*).

*Circular Dichroism of Adenylosuccinate Lyase.* A Jasco J-710 spectropolarimeter was used to carry out circular dichroism experiments. Each enzyme sample (0.1−0.15 mg/mL), in 20 mM potassium phosphate buffer, pH 7.0, containing 20 mM NaCl, was run in triplicate and averaged. Protein concentrations were determined using the BioRad protein assay, based upon the method of Bradford with wild-type adenylosuccinate lyase used as the protein standard (*19*). Ellipticity as a function of wavelength was measured from 250 to 200 nm for each sample, in 0.1 nm increments. Individual samples were scanned 5 times and averaged, and the background buffer spectrum was subtracted out. The mean molar ellipticity was then calculated using the equation: $[\theta] = \theta/10nCl$, where $\theta$ is the measured ellipticity in millidegrees, $C$ is the molar concentration of enzyme subunits, $n$ is the number of residues in 1 subunit (437, which includes the 6-His tag), and $l$ is the path length in centimeters.

*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 (*20*). 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, using 60 $\mu$M adenylosuccinate. Specific activity, in the direction of AMP formation, is defined as micromoles of adenylosuccinate converted per minute per milligram of enzyme.

The $k_{cat}$ and $K_m$ values were determined by varying the concentration of substrate present. The data were analyzed from Lineweaver−Burk plots, and error estimates were obtained from Sigma Plot. 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 282 nm, the rate in the reverse direction was monitored at 290 nm, using the difference extinction coefficient of 4050 $M^{-1}$ $cm^{-1}$.

*Molecular Mass Determination of the Lys[268] Mutant Enzymes.* A miniDAWN laser photometer (Wyatt Technology Corp., Santa Barbara, CA) was used to determine the molecular mass of the Lys[268] mutant enzymes. The molecular mass of each enzyme was evaluated at a protein concentration of approximately 0.2 mg/mL in 20 mM potassium phosphate, pH 7.0, in the presence of 20 mM NaCl. The protein concentrations were determined by the BioRad protein assay. Data were collected at a laser wavelength of 690 nm and analyzed using ASTRA software for Windows. The theory and operation of this instrumentation have been previously described (*21*).

*Molecular Mass Determination of the Glu[275] Mutant Enzymes.* Native polyacrylamide gel electrophoresis was used to determine the oligomeric state of the Glu[275] mutant enzymes. The gel compositions were 0.06 M HCl, 0.07 M Tris, 0.115% (v/v) *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, 0.2 mg/mL ammonium persulfate, and appropriate volumes of $H_2O$, and 37.5:1 acrylamide:bisacrylamide to make gels with final acrylamide concentrations ranging from 5 to 8%. Electrophoresis buffer consisted of 20 mM thioglycolic acid and 20 mM sodium barbital, pH 7.0. The gels were pre-run at 25 mA for 90 min at 4 °C before the protein samples, molecular mass standards, and bromophenol blue tracking dye were loaded. Electrophoresis was carried out at 25 mA (4 °C) until the tracking dye was almost to the end of the gel. The molecular masses of wild-type and the Glu[275] mutant enzymes were determined by the method of Hedrick and Smith (*22*). Ovalbumin (45 kDa), bovine serum albumin (67 kDa), alcohol dehydrogenase (141 kDa), and ferritin (450 kDa) served as molecular mass standards.

*Synthesis of [2-$^3$H]-Adenylosuccinate.* The preparation of [2-$^3$H]-adenylosuccinate was carried out enzymatically, starting with fumarate and [2-$^3$H]-AMP, using a modification of a previously described method (*12*). An ammonium bicarbonate gradient (10−300 mM) was substituted for ammonium acetate in eluting the DEAE-cellulose column; and a Dowex 50 column (BioRad AG 50W-X4, 100−200 mesh, hydrogen form) was utilized for desalting the product. The final product was obtained in 72% yield, and had a specific radioactivity of $6.20 \times 10^{11}$ cpm/mol.

*[2-$^3$H]-Adenylosuccinate Binding Experiments.* Using ultrafiltration, the ability of inactive mutant enzymes to bind adenylosuccinate was determined. The experiment was carried out under identical conditions as described in Lee et al. (*12*). H68A adenylosuccinate lyase was used as a positive control for binding, as this inactive mutant enzyme is known to retain its binding ability. To determine the amount of bound adenylosuccinate, the concentration of free adenylosuccinate was subtracted from the total adenylosuccinate concentration.

*Intersubunit Complementation of Glu[275] and Lys[268] Mutant Enzymes.* Combinations of H141Q, H89Q (*8*), E275Q, and K268Q adenylosuccinate lyases were evaluated for intersubunit complementation. For the E275Q & H89Q pair, each enzyme (1.4 mg/mL) was incubated separately at 25 °C for 0.5 h in 20 mM potassium phosphate, containing 20 mM NaCl, pH 7.0. Equal volumes of each enzyme pair were mixed, with the final concentration of each mutant enzyme being 0.7 mg/mL. Reactivation was followed over time using the "standard assay".

For the K268Q & H89Q pair, each enzyme (5.8 mg/mL) was incubated at 25 °C for 1 h in 20 mM potassium phosphate, containing 20 mM NaCl, 0.83 M urea, pH 7.0. The enzymes (equal volumes) were then mixed and diluted 5-fold, yielding final concentrations of 0.6 mg/mL for each enzyme and 0.16 M for urea. Activity restoration at 25 °C was assayed over time.

Finally, for complementation of either K268Q or E275Q by H141Q, slow freezing and thawing was utilized. Each enzyme (1.4 mg/mL), in its storage buffer of 20 mM sodium phosphate, containing 20 mM NaCl, pH 7.0, was mixed and slowly frozen at −20 °C. The pair was then slowly thawed at 25 °C and assayed over time for complementation. Wild-type adenylosuccinate lyase, treated identically to the complementation pairs, served as the control in all cases.

*Homology Modeling of Adenylosuccinate Lyase from Bacillus subtilis.* Homology modeling to create the adenylosuccinate lyase of *Bacillus subtilis* structure was accomplished through the use of the Swiss-Pdb Viewer 3.7 program (*23*−*25*). The crystal structure of adenylosuccinate lyase of *Thermotoga maritima* (PDB 1c3c) served as the template (*15*). Using the Magic Fit option of Swiss-Pdb Viewer, the *Bacillus subtilis* adenylosuccinate lyase sequence was threaded onto the template. The initial model was then submitted to the SWISS-MODEL Protein Modeling Server (*26*) in the "Optimize" mode, which generated a refined, energy-minimized structure using ProMod II and Gromos96. The electron density of the crystal structure was missing for residues 258−262, an area of high mobility. This loop was reconstructed in the model by the ProModII program, which scans the Protein DataBank for suitable fragments. Adenylosuccinate was subsequently manually docked into the energy-minimized structure using the InsightII molecular modeling program (Biosym/MSI, San Diego, CA). Manual substrate docking, guided by biochemical data, was performed as the active site is large, and allows several energetically equivalent docked structures.

## RESULTS

*Activity and Purity of the Lys[268] and Glu[275] Mutant Adenylosuccinate Lyases.* Lys[268] is a completely conserved residue that falls within the "signature sequence" of the fumarase superfamily. Enzymes in which this lysine was mutated to alanine, glutamate, glutamine, and arginine were constructed, overexpressed, and purified. The homogeneity of the resultant proteins was assessed by SDS−PAGE gels, and each enzyme found to be pure and of the correct subunit molecular mass. The specific activity, defined as the micromoles of adenylosuccinate converted per minute per milligram of protein, was determined for each mutant enzyme. The activities for the mutant enzymes were found to be 0.055, 0.00069, and 0.00028 $\mu$mol min$^{-1}$ mg$^{-1}$ for K268R, K268Q, and K268E, respectively, compared to a wild-type value of 1.56 $\mu$mol min$^{-1}$ mg$^{-1}$. The K268A enzyme had no detectable activity. As evidenced by the significantly decreased activities, mutations at position 268 markedly impact enzyme function.

Glu[275] is a highly conserved residue that is at the end of the "signature sequence" and is in close proximity to the well-conserved general acid/base His[141] on a neighboring subunit. Mutations of Glu[275] to alanine, aspartate, glutamine, and lysine were constructed, overexpressed, and purified. These mutant enzymes were expressed well and are pure as determined by SDS−PAGE. Attempts were made to measure the specific activity of these enzymes, but all lacked detectable activity. The lower limit of detectable activity is approximately $5 \times 10^{-5}$ $\mu$mol min$^{-1}$ mg$^{-1}$. Based on the lack of activity observed in all of these mutant enzymes, Glu[275] appears to be an essential amino acid of adenylosuccinate lyase.

*Circular Dichroism Spectroscopy of Wild-Type, Glu[275], and Lys[268] Mutant Adenylosuccinate Lyases.* Circular dichroism was utilized to determine whether the reduced, or nondetectable, activity of the mutant enzymes was caused

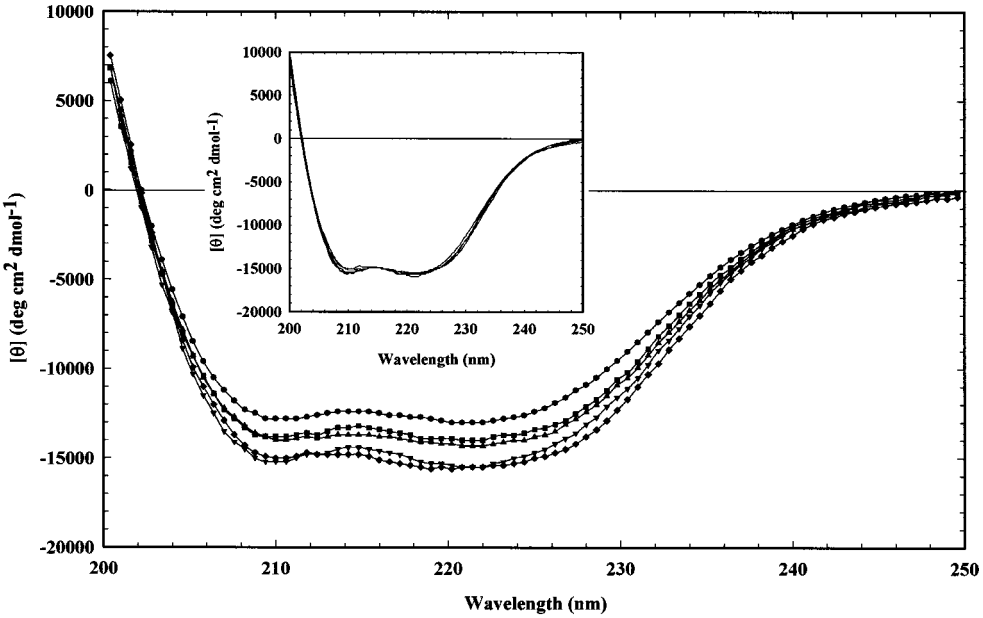


FIGURE 2: Circular dichroism spectra of the wild-type and Glu[275] mutant enzymes in the far-UV region. Each spectrum has been corrected for the background contributed by buffer and is normalized to the same enzyme concentration. Protein samples: (◆) wild-type, (▼) E275A, (●) E275D, (■) E275K, (▲) E275Q. The inset shows the spectra of the wild-type and Glu[275] mutant adenylosuccinate lyases normalized to [$\theta$] at 215 nm in order to compare their relative shapes.

by major changes in secondary structure. Enzymes in this superfamily have relatively high amounts of α-helical structure, and yield CD spectra typical of such, with minima at 208 and 222 nm. The spectra of the Lys[268] mutant enzymes are not markedly different from that of wild-type; the shapes and relative intensities of all the spectra are similar, with prominent α-helical features (data not shown). Figure 2 shows the spectra of the Glu[275] mutant enzymes. The inset shows the spectra normalized to $\theta$ at 215 nm, and reveals that the Glu[275] mutants are not appreciably different from wild-type enzyme with respect to their overall shape. However, they do vary somewhat in their relative intensities from the spectra of wild-type enzymes, which suggests the mutant enzymes' α-helix content is slightly lower than that of wild type.

*Light Scattering of Wild-Type and Lys[268] Mutant Enzymes.* Adenylosuccinate lyase has a subunit molecular mass of 50 kDa, and the biologically active form of the enzyme, a tetramer, has been shown to have a molecular mass of 200 kDa (*12*, *14*). To evaluate the possibility that the mutations at position 268 have affected the oligomeric state of the enzyme, light scattering was used. The absolute molar masses were determined for wild type, K268A, K268E, K268Q, and K268R, and found to be 195 ± 23, 186 ± 42, 228 ± 41, 217 ± 16, and 215 ± 27 kDa, respectively. These masses are all similar to wild type and are consistent with each enzyme being a tetramer.

*Molecular Mass Determination of the Glu[275] Mutant Enyzmes by Electrophoresis.* To investigate whether the inactivity of the Glu[275] mutant enzymes was due to a major change in the oligomeric state of the enzyme, their molecular masses were determined. Light scattering proved to be an unsatisfactory method because of enzyme instability; thus, native polyacrylamide gel electrophoresis was conducted, using 5−8% polyacrylamide gels. Figure 3 shows an 6% gel as representative. Based upon relative mobilities ($R_M$) in the various percentage gels, the following molecular masses

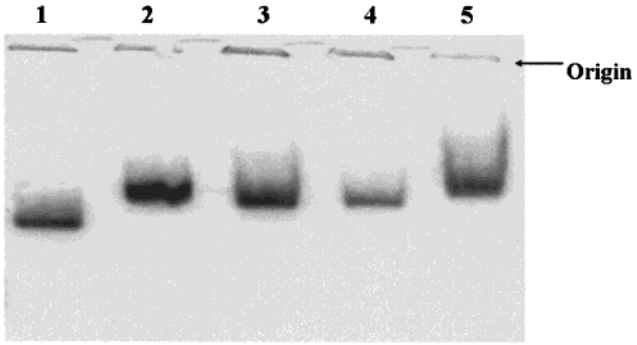


FIGURE 3: Polyacrylamide gel (6%) electrophoresis under native conditions was set up with the proteins migrating toward the anode. The electrophoresis buffer consisted of 20 mM sodium barbital and 20 mM thioglycolic acid, pH 7.0. Electrophoresis was carried out at 4 °C at 25 mA. Lanes: (1) wild-type adenylosuccinate lyase, (2) E275A, (3) E275D, (4) E275Q, (5) E275K

of the samples were calculated: wild-type, 202 kDa; E275A, 264 kDa; E275D, 187 kDa; E275K, 214 kDa; E275Q, 204 kDa. The Glu[275] mutant enzymes thus appear to exist in the tetrameric state. However, when compared to wild-type adenylosuccinate lyase on the gels, the mutant enzymes run slightly slower than wild-type, perhaps indicating that these enzymes differ from wild-type in their overall shape.

*Kinetic Parameters of Wild-Type and K268R Enzymes.* Of the mutations constructed, only the positively charged K268R enzyme had sufficient activity to characterize kinetically. The kinetic parameters were evaluated both in the direction of AMP formation ("forward reaction") and in the direction of adenylosuccinate formation ("reverse reaction").

The kinetic data in the forward direction for both wild-type and K268R adenylosuccinate lyase are summarized in Table 1. In this conservative mutation, the $K_m$ for adenylosuccinate is not significantly different from that of wild-type enzyme. However, the $V_{max}$ is 28-fold lower in the K268R enzyme than it is in wild-type, and the catalytic efficiency

Table 1: Summary of Wild-Type and K268R Adenylosuccinate Lyase Kinetic Parameters in the Direction of AMP Formation

| enzyme | $V_{max}$[a] ($\mu$mol min$^{-1}$ mg$^{-1}$) | $K_{m,adenylosuccinate}$ ($\mu$M) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) |
|---|---|---|---|---|
| wild-type | $1.56 \pm 0.19$ | $3.46 \pm 0.44$ | $1.30 \pm 0.16$ | $3.75 \times 10^5$ |
| K268R | $0.06 \pm 0.003$ | $5.56 \pm 0.50$ | $0.05 \pm 0.003$ | $0.09 \times 10^5$ |

[a] The specific activities for K268Q, K268E, and K268A were measured at 25 °C, in 50 mM HEPES buffer, pH 7.0, in the presence of 60 $\mu$M adenylosuccinate. K268Q and K268E had specific activities of 0.00069 and 0.00028 $\mu$mol min$^{-1}$ mg$^{-1}$, respectively. The K268A enzyme had no detectable activity; the lower limit of detectability is approximately $5 \times 10^{-5}$ $\mu$mol min$^{-1}$ mg$^{-1}$.

($k_{cat}/K_m$) of this particular mutant enzyme is only 2% that of wild-type.

A summary of the kinetic data in the reverse direction for both wild-type and K268R adenylosuccinate lyase is found in Table 2. In this direction, the $K_m$s for both AMP and fumarate remain unaltered from that of wild-type. In K268R, $V_{max}$ and the catalytic efficiency are reduced 12- and 7.5-fold from wild-type enzyme, respectively. From these results, it appears that with a positively charged residue at position 268, the affinity for substrate is relatively unaffected, while both $V_{max}$ and the catalytic efficiency are reduced.

*[2-$^3$H]-Adenylosuccinate Binding Studies of the Glu[275] and K268A Mutant Adenylosuccinate Lyases.* To better assess the function of the completely inactive mutant enzymes, their affinity for substrate was evaluated by radioactive binding studies. Previously, this method was successfully used in this laboratory to measure the binding of adenylosuccinate by other mutant adenylosuccinate lyases (*12*). The highest $K_d$ that can be detected by this method is about 200 $\mu$M.

Since adenylosuccinate lyase is a one-substrate enzyme, the binding of adenylosuccinate, without conversion to AMP and fumarate, can only be tested for enzymes that are catalytically inert. Of the Lys[268] mutant enzymes, only K268A lacked detectable activity, and was therefore a good candidate to study binding. No binding of radioactive adenylosuccinate was observed for K268A adenylosuccinate lyase. These data, combined with kinetic data for the K268R enzyme, suggest that a positively charged amino acid at position 268 is necessary for binding of the negatively charged substrate.

Since none of the mutant Glu[275] adenylosuccinate lyases has activity, their binding abilities were tested. Again, no binding of [2-$^3$H]-adenylosuccinate by any of these mutant enzymes was observed, even by the conservative glutamate to aspartate mutant. Based on these results, the role of Glu[275] in binding adenylosuccinate appears critical.

*Intersubunit Complementation of Lys[268] and Glu[275] Mutant Enzymes.* Adenylosuccinate lyase is a multimeric protein, with residues from more than one subunit contributing to the active site. When mixtures are made of subunits containing mutations of different amino acids on distinct subunits, it is possible to generate wild-type-like active sites and restore activity to the enzyme; this phenomenon is known as intersubunit complementation (*12, 14, 27*). From these experiments, the number of subunits contributing residues to the active site may be determined. Adenylosuccinate lyase has been shown to exhibit complementation. In theory, if there is complete randomization of the subunits, the activity is expected to be restored to 25% of that of wild-type enzyme (*12, 14*).

Previously, mutant enzymes with replacements for His[141] and His[89], each on different subunits, were shown to exhibit such complementation with regeneration of approximately 17% of wild-type activity (*14*). In these previous experiments, the maximum activity was achieved by simply mixing the two mutant enzymes at room temperature and assaying for activity as a function of time, generally over 2 h.

From the crystal structure and model of adenylosuccinate lyase, it appears that both Lys[268] and Glu[275] are contributed to the active site from a third subunit, distinct from the subunits contributing either His[89] or His[141]. Thus, E275Q and K268Q were tested against H89Q and H141Q adenylosuccinate lyases for restoration of activity.

The greatest reactivation was observed when testing H89Q against either K268Q or E275Q (Figure 4). H89Q and E275Q yielded 16% wild-type specific activity upon mixing the enzymes at room temperature and assaying over time. H89Q and K268Q also exhibited significant reactivation, reaching 13% of wild-type activity. However, for the latter pair, this level of reactivation was only reached after prior incubation with 0.83 M urea.

Complementation was less impressive when testing H141Q with K268Q or E275Q, yielding only 6.7% and 6.2% of wild-type specific activity, respectively. To achieve this level of restoration, the individual enzymes, in sodium phosphate buffer, were slowly frozen, and then slowly thawed. After thawing, the enzyme pairs were assayed for activity, with maximal activity occurring within 15 min of thawing. Less activity was regained if these experiments were conducted at room temperature as a function of time. Despite efforts to improve these results via extended cold incubation, urea dissociation, or the addition of dithiotheitol, no greater levels of activity restoration were achieved.

Finally, if Lys[268] and Glu[275] are located together on a third subunit, as the structure implies, there should be no complementation between these two pairs. Reactivation was tested by mixing the two enzymes at room temperature and assaying for activity as a function of time. As expected, no complementation was observed upon testing the K268Q and E275Q pair (Figure 4).

## DISCUSSION

The results of replacing two residues in the region of the signature sequence of adenylosuccinate lyase, Lys[268] and Glu[275], show that both are important for catalytic activity. Lys[268] was mutated to Arg, Gln, Glu, and Ala, and each

Table 2: Summary of Wild-Type and K268R Adenylosuccinate Lyase Kinetic Parameters in the Direction of Adenylosuccinate Formation

| enzyme | $V_{max}$ ($\mu$mol min$^{-1}$ mg$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $K_m$ (mM) AMP | $K_m$ (mM) fumarate | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) AMP | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) fumarate |
|---|---|---|---|---|---|---|
| wild-type | $3.27 \pm 0.14$ | $2.69 \pm 0.11$ | $0.06 \pm 0.01$ | $1.54 \pm 0.09$ | $4.13 \times 10^4$ | $1.74 \times 10^3$ |
| K268R | $0.26 \pm 0.01$ | $0.22 \pm 0.01$ | $0.10 \pm 0.01$ | $0.91 \pm 0.05$ | $0.22 \times 10^4$ | $0.23 \times 10^3$ |

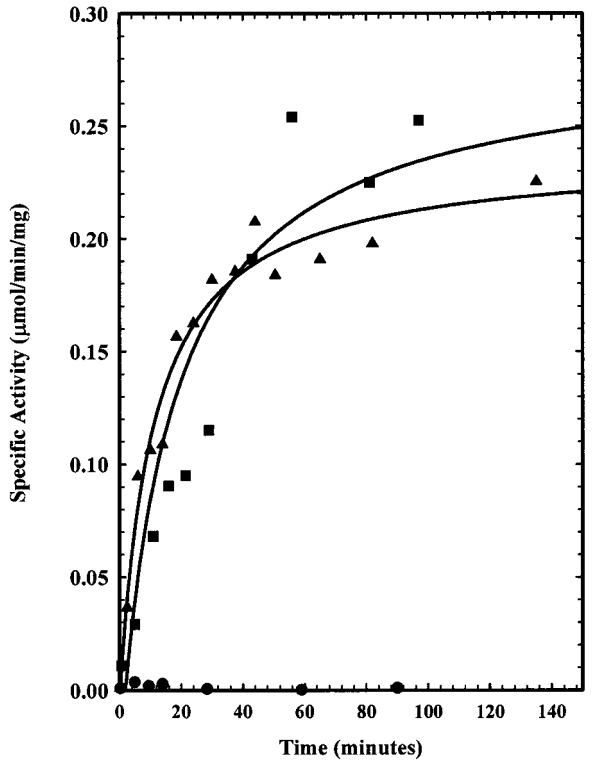


FIGURE 4: In vitro complementation studies. (■) E275Q + H89Q, (●) K268Q + E275Q: Each mutant was incubated separately before mixing at time zero. The final mixture contained 0.7 mg/mL of each enzyme. (▲) K268Q + H89Q: Each enzyme was separately incubated with 0.83 M urea, mixed, diluted, and assayed over time. The final enzyme concentration was 1.2 mg/mL, and the final urea concentration was 0.16 M. Wild-type enzyme has a specific activity of 1.56; the E275Q + H89Q and K268Q + H89Q pairs have a final average specific activity of 0.25 and 0.21, respectively.

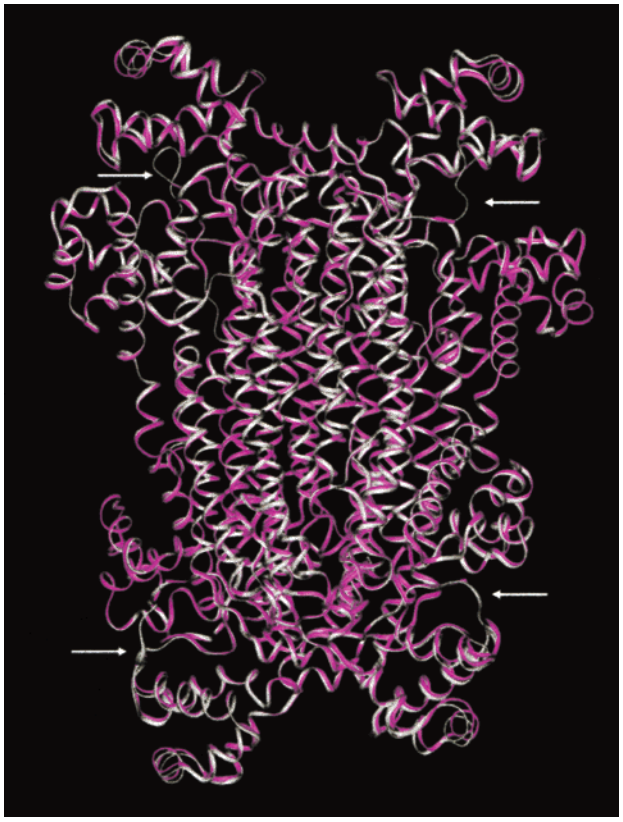

FIGURE 5: Overlay of the homology model of *B. subtilis* adenylosuccinate lyase (shown in white) and the crystal structure of *T. maritima* adenylosuccinate lyase (shown in pink). White arrows indicate the area of greatest deviation between the two structures: The highly mobile residues 258−262 are absent in the crystal structure, but are included in the homology model of the *B. subtilis* enzyme.

resultant enzyme was characterized. These mutations allowed for the evaluation of the role of charge, size, and hydrophobicity in the local region. K268R, which retains a positive charge at this position, was the most active of the $Lys^{268}$ mutant enzymes. This enzyme still has the ability to bind substrate, with the measured $K_m$s for all substrates being very close to those of wild-type enzyme. Furthermore, the $V_{max}$ for K268R is as high as 4% and 8% of the wild-type $V_{max}$ in the forward and reverse directions, respectively. The decrease in the $V_{max}$ of K268R as compared with wild type is probably attributable to distortions in the active site and/or substrate orientation caused by the presence of the larger amino acid, arginine. K268Q and K268E have marginal activity, which was insufficient for the determination of the Michaelis constants. However, the neutral glutamine mutant has a somewhat higher specific activity than that of the negatively charged glutamate enzyme, consistent with charge repulsion between the negatively charged substrate and a glutamate at position 268. K268A lacks detectable activity, implying that both polarity and size are key in this position. Furthermore, direct binding experiments show that the K268A enzyme has no measurable affinity for radioactive adenylosuccinate.

These results indicate that a positive charge at position 268 is critical for the binding of substrate; only the positively charged arginine mutant enzyme retains wild-type-like $K_m$ values. Despite the charge repulsion, the detection of activity, albeit low, in the glutamate mutant, suggests that the size of the residue at this location is also important. Circular dichroism revealed that no appreciable change in secondary structure had occurred in any of the $Lys^{268}$ mutant enzymes, and therefore these observations are not reflective of marked changes in enzyme conformation. We propose that $Lys^{268}$ interacts with a major determinant of substrate binding.

$Glu^{275}$ is a residue of particular interest based not only upon its conservation and proximity to the active site, but also on its close proximity to the proposed general base $His^{141}$. Furthermore, this $Glu^{275}$−$His^{141}$ interaction is at a subunit interface. Glutamate 275 was mutated to Ala, Asp, Gln, and Lys. Not one of the homogeneous $Glu^{275}$ mutant enzymes has detectable activity, revealing the essential nature of this residue. In addition, the substrate affinity of these inactive enzymes was evaluated by their ability to bind radioactive adenylosuccinate; none was able to bind substrate measurably.

Physical characterization of the $Glu^{275}$ mutant enzymes by native polyacrylamide gel electrophoresis and circular dichroism suggests that, although the enzyme is still a tetramer, a small change in conformation has occurred. It is possible that the active site residues are improperly positioned for both catalysis and binding.

Greatly facilitating the interpretation of our results is the availability of the crystal structure of adenylosuccinate lyase from *Thermatoga maritima*. Adenylosuccinate lyases from *B. subtilis* and *T. maritima* have 49% sequence identity and 23% high sequence similarity (based on amino acid physico-

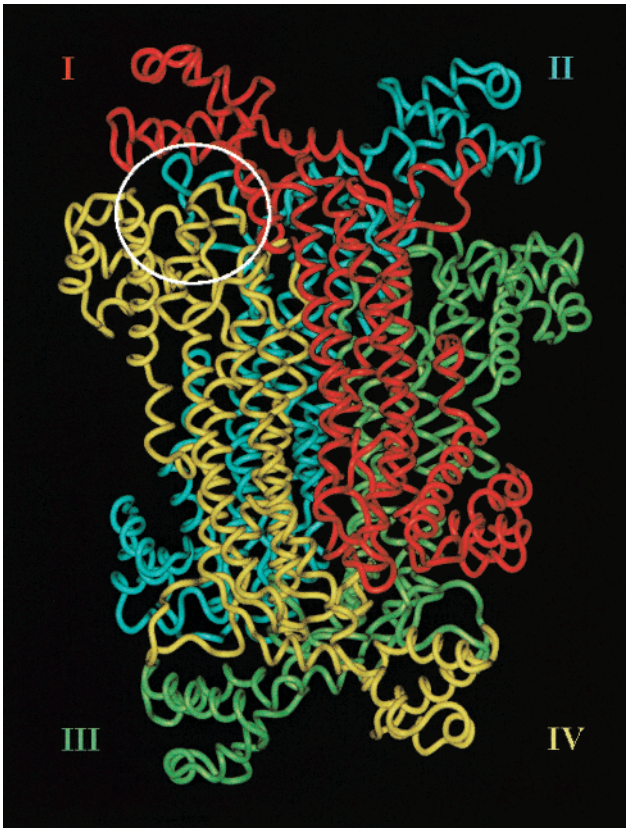


FIGURE 6: Homology model of adenylosuccinate lyase of *Bacillus subtilis* based upon the crystal structure of *Thermatoga maritima* adenylosuccinate lyase. Each individual subunit is color-coded. The white encircles one active site, shown in greater detail in Figure 7.

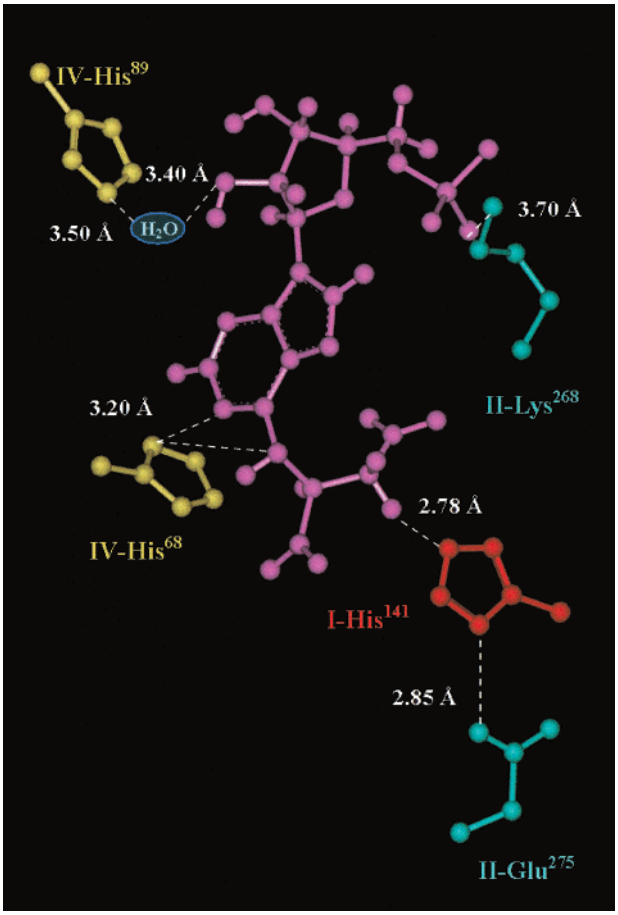


FIGURE 7: One model of adenylosuccinate docked into *B. subtilis* adenylosuccinate lyase. His[141] and His[68] are positioned to serve as the general base and acid, respectively. His[89] interacts with a ribose hydroxyl through a water. Lys[268] is in close proximity to the negatively charged phosphate group of the substrate, while Glu[275] interacts closely with His[141].

chemical properties) using the ClustalW program (*28*). The enzymes in the fumarase superfamily, to which adenylosuccinate lyase belongs, all share a high degree of structural conservation. Based on this similarity, a homology model of adenylosuccinate lyase of *B. subtilis* was constructed using the *T. maritima* enzyme as the template, threading on the sequence of the *B. subtilis* enzyme, and then carrying out an energy minimization (see Experimental Procedures). An overlay of the crystal structure of the *T. maritima* enzyme and the homology model of the *B. subtilis* enzyme is shown in Figure 5. The extent of coincidence of the two structures is remarkable. All amino acids thus far found to contribute to function are at the same position in both enzymes. The greatest deviation between the two structures lies in the loop region, specifically residues 258−262 on each subunit, marked by the white arrows; the electron density in this region was not seen in the crystal structure of the *T. maritima* enzyme, presumably because these residues are highly mobile in the apoenzyme. Through ProModII at Swiss-Model, which scans the Protein DataBank for appropriate fragments, these residues were included in the final model. An rmsd value, excluding the 258−262 region, was calculated using the backbone atoms of each structure and found to be 0.15 Å; thus, the model generated should provide a valid depiction of adenylosuccinate lyase of *B. subtilis*. The homology model alone, with each subunit color-coded, is shown in Figure 6. The white circle highlights one of the four active sites. From Figure 6, it is apparent that three subunits contribute residues to the active site. Other members of the superfamily (argininosuccinate lyase, fumarase, and $\delta$-crystallin) have also been shown to have active sites composed of amino acids from three subunits (*29*−*32*).

Using the newly generated model for *B. subtilis* adenylosuccinate lyase, and all the available biochemical data, adenylosuccinate was manually docked into the active site as shown in Figure 7. (Although the active site is large and the substrate can be docked in more than one manner that is energetically reasonable, it is shown in the orientation which is most consistent with the biochemical data.) All van der Waals interactions are reasonable. In this docking scenario, His[141] is well positioned to serve as the general base, with the $\epsilon$-N being only 2.78 Å from the $\beta$-H of the succinyl moiety. Located 3.20 Å from N1 or 6-$NH_2$ of the adenine ring, the $\epsilon$-N of His[68] makes this residue well positioned to function as the general acid. Of relevance to the work described in this paper, the $\epsilon$-N of the positively charged Lys[268] is within 3.7 Å of a negatively charged phosphoryl oxygen of the substrate, allowing for significant electrostatic attraction. Previous work in our laboratory (*14*) has also shown that His[89] is involved in binding the AMP portion of substrate, with the $K_m$ of AMP being 13-fold greater in the H89Q mutant enzyme than it is in wild-type. We had previously proposed that this residue interacts with the phosphate group of substrate. In light of the inability of the neutral K268A mutant enzyme to bind substrate, it now seems more likely that the positively charged Lys[268] interacts

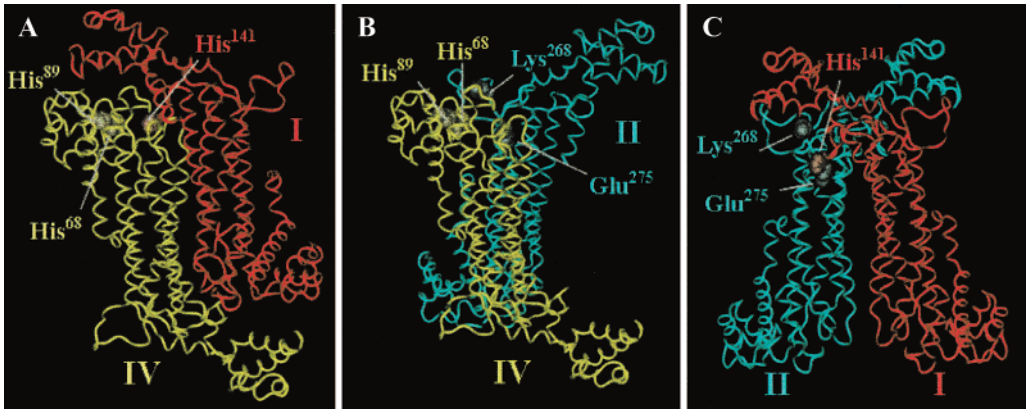


FIGURE 8: Three different types of subunit interactions found within the adenylosuccinate lyase tetramer. Subunit numbering is the same as given in Figure 6. (A and B) Both subunits I and IV and subunits II and IV have relatively strong interactions throughout the central α-helix bundle. (C) The fewest interactions are found between subunits I and II, localized near the active site.

with the major binding determinant, phosphate. $His^{89}$ is still positioned, though, to interact with another region of the AMP portion of the molecule: the ribose hydroxyls. The crystal structure reveals that there is a water molecule close to the $\epsilon$-N of $His^{89}$, and in the enzyme model containing docked substrate, this water is also in close proximity to the 2′-O of the ribose. It is likely that this water could facilitate a binding interaction between a ribose hydroxyl and $His^{89}$. Homology models, in which the $Lys^{268}$ mutations were simulated, further support this idea. Each of the replacement residues at position 268 was no longer in close proximity to the phosphate group; K268A was the worst with its side chain being located almost 7 Å from the phosphoryl oxygens. Even K268Q, which roughly approximates the size of a lysine, was slightly rotated, and was 4.7 Å from the closest phosphoryl oxygen. Replacement of the positively charged lysine by the negatively charged glutamate would lead to electrostatic repulsion of the negatively charge phosphate moiety; in that case, the glutamate at position 268 appeared to be involved in hydrogen bonding with the backbone of another subunit. Therefore, we propose that $Lys^{268}$ is a major binding residue that interacts with the phosphate group of adenylosuccinate. Our experimental data, combined with the homology models, suggest that both size and charge are critical at this position for productive interaction with substrate.

Both the crystal structure of the *T. maritima* enzyme and the homology model of the *B. subtilis* enzyme reveal a very close interaction between $Glu^{275}$ and $His^{141}$. Measuring from the $\delta$-N of $His^{141}$ to the $\delta$-O of $Glu^{275}$, these residues are separated by only 2.85 Å, a distance sufficient for hydrogen bonding and for $Glu^{275}$ to influence the orientation of $His^{141}$ (Figure 7). One explanation for the inactivity of the $Glu^{275}$ mutant enzymes is that the $His^{141}-Glu^{275}$ interaction is disrupted. All the $Glu^{275}$ mutant enzymes were modeled, and none of the replacement residues was close enough to $His^{141}$ for hydrogen bonding interactions to occur. Even in the conservatively mutated E275D enzyme, the distance between the $\delta$-N of $His^{141}$ and a carboxylate oxygen of $Asp^{275}$ is 4.06 Å.[2] It is possible that the role of $Glu^{275}$ is to orient $His^{141}$ for catalysis; without such positioning, $His^{141}$ would be unable to abstract the $\beta$-H from substrate. After proton abstraction, $Glu^{275}$ may also play a role in charge stabilization of the now protonated $His^{141}$. Our models suggest that such interactions would no longer be effectively taking place in the mutated $Glu^{275}$ enzymes. The role of such close interactions between histidine and either glutamate or aspartate has been discussed in the literature for enzymes such as phosphoglucose isomerase (*33*), glucose-6-phosphate dehydrogenase (*34*, *35*), phosphatidylinositol-specific phospholipase (*36*), and ribonuclease A (*37*). In the majority of these interactions, the role of a His-Glu or His-Asp catalytic dyad involves correctly orienting histidine, moderating the p*K* of histidine, maintaining histidine in the correct tautomeric form, and/or charge stabilization in the case of a general base. Based upon the observed minor conformational changes, it is possible that the $Glu^{275}-His^{141}$ interaction, located at a subunit interface, may also play a role in maintaining enzyme conformation.

The lack of substrate binding in the $Glu^{275}$ mutant adenylosuccinate lyases was initially perplexing, as this residue does not appear to be in direct contact with adenylosuccinate. However, perturbing the $Glu^{275}-His^{141}$ interaction could alter the conformation of the active site sufficiently so that substrate is no longer able to bind. Alternatively, adenylosuccinate lyase is a dynamic molecule, and a mutation at position 275 might "lock" the enzyme into a conformation not amenable to substrate binding.[2]

The $Lys^{268}$ and $Glu^{275}$ mutant adenylosuccinate lyases also provide important information with respect to subunit contributions to the active site. In vitro complementation experiments may be used to determine which subunits are contributing residues to the active site (*27*). It has previously been demonstrated that $His^{68}$ and $His^{89}$ come from one subunit, while $His^{141}$ is contributed by a second subunit (*12*, *14*). Using H89Q and H141Q, E275Q and K268Q could be tested against two distinct subunits. In theory, if there is complete randomization of subunits, restoration of wild-type activity to 25% may be expected. H89Q and E275Q exhibited complementation comparable to levels exhibited by $His^{141}$ and $His^{89}$; up to 16% of wild-type activity was regained (Figure 4).[3] That urea was needed before significant reactivation was observed with H89Q and K268Q suggests that this particular enzyme pair is difficult to dissociate from the tetrameric state. Using H141Q and either K268Q or E275Q,

[2] Alternatively, if $His^{141}$ is positioned in the enzyme model so that it can form a hydrogen bond with aspartate or glutamine at position 275, the critical $His^{141}$ is distorted from its original position.

the maximum reactivation never exceeded 7% of wild-type activity. Nonetheless, the fact that complementation was exhibited by both K268Q and E275Q when tested against either H141Q or H89Q provides the first functional evidence that a third subunit contributes residues to the active site of adenylosuccinate lyase.

One possible explanation for the minimal complementation between certain pairs lies in the arrangement of subunits within the tetramer. In general, to observe any reactivation, the individual subunits must first dissociate and then randomly reassociate. At present, the pathway of dissociation and reassociation of adenylosuccinate lyase has not been delineated. Data from our laboratory indicate the presence of a dimeric species (unpublished), but it has not been evaluated experimentally whether this enzyme reversibly dissociates to the monomer level under mild conditions. However, argininosuccinate lyase (another member of the superfamily) has been shown to dissociate to a dimer (*38*), and dimeric interactions for fumarase have been discussed (*31*). Accordingly, Figure 8 highlights the pairwise subunit interactions as extracted from the tetramer shown in Figure 6. It appears that subunits I and IV interact strongly and mutations on these subunits produce the most effective complementation, implying that neither dissociation nor reassociation presents major barriers to reactivation (Figure 8A). Subunits II and IV, too, appear to have relatively strong interactions (Figure 8B), and E275Q and H89Q exhibited striking levels of reactivation. However, when K268Q was tested against H89Q, low concentrations of urea were needed to achieve maximal levels of complementation. This requirement of urea for complementation was found for each mutant enzyme with a substitution for Lys[268], suggesting there is an impediment to dissociation. The models for each mutation at position 268 support the notion of hindered dissociation. In wild-type enzyme, Lys[268] is not involved in hydrogen bonding with its neighbors. However, in the model, most of the replacement residues are involved in new hydrogen bonding interactions, some across the subunits. Such new hydrogen bonds could conceivably hinder dissociation, necessitating the use of urea to promote complementation. These results also suggest that the region surrounding Lys[268] may be involved in mediating subunit interactions. Finally, the weakest subunit interaction appears to exist between subunits I and II (Figure 8C); in fact, the region near the active site provides almost the only locus of interaction. Complementation between these pairs was much less effective than in any of the other pairs. We propose that the limitation in complementation in this case lies in reassociation of the tetramer. Since the subunit interaction involves such a small region between subunits I and II, these mutated pairs cannot properly reassociate, resulting in poor complementation.

In conclusion, this work has demonstrated the important roles of the residues of adenylosuccinate lyase in the "signature sequence" region. Lys[268] appears to play a critical role in binding substrate, probably interacting with the phosphate group of the AMP portion of substrate. Glu[275] is an essential amino acid that has a close interaction with His[141] in the crystal structure of adenylosuccinate lyase. It is likely that Glu[275]−His[141] constitutes a catalytic dyad that is also necessary for substrate binding. In addition, Glu[275] may assist in mediating proper subunit interactions within the tetramer. Finally, intersubunit complementation not only has provided the first functional evidence of a third subunit contributing residues to the active site in adenylosuccinate lyase, but also has offered insight into the roles of specific subunit interactions.

[3] The substantial restoration of activity observed (up to 16% of the theoretical maximum of 25% of wild-type activity) upon mixing inactive H89Q with either K268Q or E275Q argues that these mutant enzymes cannot have undergone an extensive conformational change.

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