Residues Essential for Catalysis and Stability of the Active Site of Escherichia coli Adenylosuccinate Synthetase as Revealed by Directed Mutation and Kinetics*

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Examined here by directed mutation, circular dichroism spectroscopy, and kinetics are the relationships of five residues, Asp13, Glu14, Lys16, His41, and Arg131, to the catalytic function and structural organization of adenylosuccinate synthetase from Escherichia coli. The D13A mutant has no measurable activity. Mutants E14A and H41N exhibit 1% of the activity of the wild-type enzyme and 2–7-fold increases in the $K_m$ values for substrates virtually unchanged from those of the wild-type system. Mutation of Arg131 to leucine caused only a 4-fold increase in the $K_m$ for aspartate relative to the wild-type enzyme. The dramatic effects of the D13A, E14A, and H41N mutations on $k_{cat}$ are consistent with the putative roles assigned to Asp13 (catalytic base), His41 (catalytic acid), and Glu14 (structural organization of the active site). The modest effect of the R131L mutation on the binding of aspartate is also in harmony with recent crystallographic investigations, which suggests that Arg131 stabilizes the conformation of the loop that binds the $\beta$-carboxylate of aspartate. The modest effect of the K16Q mutation, however, contrasts with significant changes brought about by the mutation of the corresponding lysine in the P-loop of other GTP- and ATP-binding proteins. Crystallographic structures place Lys16 in a position of direct interaction with the $\gamma$-phosphate of GTP. Furthermore, lysine is present at corresponding positions in all known sequences of adenylosuccinate synthetase. We suggest that along with a modest role in stabilizing the transition state of the phosphotransfer reaction, Lys16 may stabilize the enzyme structurally. In addition, the modest loss of catalytic activity of the K16Q mutant may confer such a selective disadvantage to E. coli that this seemingly innocuous mutation is not tolerated in nature.

Adenylosuccinate synthetase (AMPSase) (see Ref. 1 for review) catalyzes the following reversible reaction in the presence of Mg$^{2+}$ ions: GDP + IMP + aspartate ⇌ GDP + adenylosuccinate + phosphate (P). This reaction is the first committed step in the formation of AMP from IMP on the pathway for de novo purine nucleotide biosynthesis and is an integral part of the purine nucleotide cycle in muscle (2). The reaction mechanism of AMPSase centers on 6-phosphoryl-IMP, formed putatively by the nucleophilic attack of the 6-oxyanion of IMP on the $\gamma$-phosphate of GTP. A second nucleophilic substitution reaction by the amino group of aspartate on the C-6 of 6-phosphoryl-IMP yields adenylosuccinate and P$_i$ (3). Two Mg$^{2+}$ ions are involved in the reaction mechanism (4). One Mg$^{2+}$ is in the active site, associated with the phosphate moiety of the guanine nucleotide and the N-formyl group of hadacidin, an inactive analog of aspartate (5). However, crystallographic investigations have yet to reveal the location of the second Mg$^{2+}$.

On the basis of preliminary crystal structures of ligated AMPSase, which are now complete (5, 6), several residues are in positions of putative significance to catalytic function, ligand binding, and/or structural organization of the active site. Asp13 of AMPSase hydrogen bonds with N-1 of IMP and approaches the sixth coordination site of a pentavalently coordinated Mg$^{2+}$. Asp13 is putatively a catalytic base in the abstraction of the proton from N-1 of IMP (5). Glu14 hydrogen bonds to the backbone amides 10 and 12 of the P-loop (residues 8–17 of AMPSase) and to NZ of Lys16. Glu14 may stabilize the conformation of the P-loop, provide electrostatic charge balance in the active site, and/or orient NZ of Lys16 with respect to the $\gamma$-phosphoryl group. Lys16 of AMPSase corresponds to the essential lysine of the consensus P-loop sequence GXXGK (7, 8). Lys16 interacts with the $\beta$-phosphate of GDP and/or anions (nitrate and phosphate) bound to the $\gamma$-phosphoryl site (5, 6). Lys16 putatively stabilizes the pentavalent transition state of the $\gamma$-phosphoryl group during the phosphotransfer reaction. His41 interacts with phosphate groups located at the $\beta$- and $\gamma$-phosphoryl binding sites. His41 is a putative catalytic acid in the phosphotransfer reaction (5).

Finally, Arg131, initially considered a candidate for binding aspartate (9), may stabilize the closed (ligand-bound) state of the active site by folding over and perhaps hydrogen bonding with the loop that recognizes the $\beta$-carboxylate of aspartate.

Reported here are mutations of Asp13, Glu14, Lys16, His41, and Arg131, which further probe the roles of each residue in the function of AMPSase.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strain XL1-Blue came from Stratagene, a site-directed mutagenesis kit from Amersham Corp., and restriction enzymes from Promega. The chemicals used in this study were obtained from Sigma, and $\alpha$-strain H1238 was a gift from Dr. B. Bachman (Genetic Center, Yale University).

Site-directed Mutagenesis—Recombinant DNA manipulation was performed using standard procedures (10). The mutagenic primers$^2$ in this study are 5'-TTTACCTTGGGACCCCATGAT-3' (Asp13→Ala), 5'-

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$^2$ The synthesis of the primers in this study and the DNA sequencing of the mutated plasmids were done by the Iowa State University Nucleic Acid Facility.
ACCTTTACCTCGTACCCC-3' (Glu-14 → Ala), 5'-ACGAGATATGGCTGCAG-3' (His-41 → Asn), and 5'-CCCCGTACAAAGACCCTGGG-3' (Arg-131 → Leu). (The bases in bold letters indicate the mutated sites.) Mutagenesis and isolation of the mutant cell lines were carried out according to the procedure described previously (11). The mutated plasmids were transformed into an E. coli pur A2 strain (H1238), which does not produce AMPSase, to prevent mutant protein contamination by wild-type enzyme.

Preparation and Kinetics of Wild-type and Mutant AMPSases— Mutant forms of AMPSase were purified by the procedure described previously (12). The H41N mutant has low solubility in 20 mM potassium Pi, pH 7. As a consequence, the eluent from the phenyl-Sepharose column was concentrated in 100 mM potassium Pi, pH 7, with a linear salt gradient from 0 to 1 M NaCl. Protein purity was monitored by SDS-polyacrylamide gel electrophoresis according to Laemmli (13). The concentration of purified protein was determined using the extinction coefficient for wild-type AMPSase at 280 nm (ε280 = 67.85 mM−1 cm−1), where the concentration refers to monomers. AMPSase activity was determined as described earlier (14). 3–20 μg/ml enzyme was used in assays, depending on the activity of each mutant. A GBC model 918 UV/Visible spectrophotometer equipped with a Peltier-Effect temperature controller to maintain the temperature at 25 °C was used to monitor absorbance changes at 290 nm.

Stability of the K16Q Mutant AMPSase Activity—Two samples of the K16Q mutant (0.54 mg/ml) in 40 mM Hepes buffer, pH 7.7, were incubated at either 25 or 4 °C for 2 h. At different times, 5-μl aliquots were removed and added to 1 ml of assay solution containing 150 μM GTP, 200 μM IMP, 5 mM aspartate, and 2 mM MgCl2 in Hepes, pH 7.7, and the activity was measured at 25 °C. The absorbance change at 290 nm was recorded. A parallel experiment was carried out with the wild-type enzyme for the purpose of comparison with the mutant.

In another study, urea (0.5 M) was used to evaluate enzyme stability involving the wild-type and K16Q mutant AMPSases. The enzymes were incubated with 0.5 M urea at 25 °C for different periods of time (0, 1, 2, 4, 8, and 16 min) and then added to the assay solution. Initial rates were measured at 280 nm and 25 °C in solutions containing 0.5 M urea.

### Table I

Kinetic parameters for the mutants in the P-loop region of E. coli adenylosuccinate synthetase

|           | kcat (sec−1) | Km,GTP (μM) | Km,IMP (μM) | Km,Asp (μM) |
|-----------|--------------|-------------|-------------|-------------|
| Wild type | 1.37 ± 0.12  | 22 ± 5      | 21 ± 2      | 280 ± 40    |
| D13A      | 1.92 ± 0.28* | 13.0 ± 1.4* | 13.1 ± 1.3* | 228 ± 16   |
| E14A      | 0.0220 ± 0.0035* | 31.0 ± 2.9* | 55.0 ± 6.8* | 1.40 ± 0.12* (mM) |
| K16Q      | 0.479 ± 0.099 | 23.0 ± 6.5  | 50.5 ± 20.4 | 308 ± 55   |
| H41N      | 0.00951 ± 0.00010 | 126 ± 13*  | 40.1 ± 8.3* | 1.95 ± 0.6 (mM) |
| R131L     | 1.41 ± 0.22  | 35.2 ± 3.4  | 35.5 ± 2.0  | 1.00 ± 0.03 |

a Determined at pH 7.
b 10 mM Asp was used.
c 0.6 mM GTP was used.

Fig. 1. Circular dichroism spectra of the wild-type protein (solid line) and the H41N mutant (dotted line).

Fig. 2. Putative roles of Asp13 and His41 as a catalytic base and acid, respectively, in the phosphotransferase reaction of adenylosuccinate synthetase.
20 mM Hapes, pH 7.7, 1 mM MgCl₂, 5 mM aspartate, 150 mM IMP, and 60 mM GTP.

Circular Dichroism Spectroscopy—Circular dichroism spectra were acquired at room temperature on a Jasco spectropolarimeter, model J-710. Samples (50–300 μg/ml in 10 mM potassium Pi, pH 7.0) were placed in a 1-mm cuvette, and data points were obtained from 200 to 260 nm in 0.5-nm increments. Spectra were normalized for a direct comparison.

RESULTS AND DISCUSSION

Growth of Transformed E. coli AMPSase—All transformants of the pur A² cell line (H1238) grew in the LB medium. D13A and E14A transformants, however, grew at a slow rate, comparable to that of the original pur A² cell line, which must draw its entire supply of adenine from the LB medium.

Purification of Mutant AMPSases—D13A, E14A, K16Q, and R131L mutants migrate comparably on phenyl-Sepharose and DEAE-HPLC columns to wild-type AMPSase. However, during concentration (Amicon concentrator) of the eluent from the phenyl-Sepharose column, the H41N mutant precipitated. The precipitated protein could be redissolved upon dilution or by increasing the concentration of KP, at pH 7 from 20 to 500 mM. Samples, precipitated and then redissolved, showed no loss of activity, ruling out the possibility of irreversible denaturation. All mutant proteins were more than 95% pure, as judged by SDS-polyacrylamide gel electrophoresis.

Circular Dichroism Spectroscopic Study of the Mutants—The CD spectra of D13A, E14A, K16Q, and R131L mutants are almost identical to that of wild-type AMPSase (data not shown). For these mutants, then, no global conformational change occurs as a consequence of mutation. However, the H41N mutant differs significantly from the wild-type protein in its CD spectrum (Fig. 1), indicating a large structural perturbation. The altered structure of the H41N mutant may be responsible for its low solubility in low salt buffer. Based upon...
x-ray diffraction studies of AMPsase (7), His\(^{11}\) hydrogen bonds to Asp\(^{21}\), an interaction that may stabilize the loop 42–53 in the absence of ligands. Differences in the CD spectra of wild-type and the H41N mutant may stem from conformational differences in this loop structure in the absence of ligands.

**D13A Mutant**—The D13A mutant shows no activity using the conventional assay, even with 1 mg/ml protein. Considering the sensitivity of this technique (approximately \(1 \times 10^{-4}\) A change/min at 290 nm), the activity must be less than 0.001% wild-type AMPsase. However, GTP and IMP quench the intrinsic fluorescence of the mutant, indicating that substrates bind to the D13A mutant. Crystal structures have revealed a hydrogen bond between the side chain of Asp\(^{13}\) and N-1 of the IMP (5). Asp\(^{13}\), then, may abstract the proton from N-1 to generate the 6-oxyanion of IMP, the putative nucleophile in the attack on the \(\gamma\)-phosphorus atom of GTP (Fig. 2). The complete loss of activity due to the D13A mutation is entirely consistent with an essential catalytic role for Asp\(^{13}\).

**E14A Mutant**—The E14A mutant exhibits greatly reduced activity. The \(k_{cat}\) of E14A is too low to be measured with confidence at pH 7.0. Thus, assays were performed at pH 7.0, where the \(k_{cat}\) of wild-type AMPsase increases by 40% and the \(K_m\) values decrease (Table I). At pH 7.0 the E14A mutant had a \(k_{cat}\) of 0.022/s (approximately 1% that of the wild-type enzyme at pH 7.0), and the \(K_m\) values for substrates are 3–6-fold higher than those of the wild-type enzyme (Table I). Given that Glu\(^{14}\) makes hydrogen bonds that stabilize the P-loop in *E. coli* AMPsase (7), the dramatic fall-off in \(k_{cat}\) of the E14A mutant may be due to a conformational perturbation on Asp\(^{13}\), which, as noted above, is a putative catalytic base. Alternatively, Glu\(^{14}\) may be essential to the electrostatic charge balance in the active site. In crystal structures, Glu\(^{14}\) makes a salt link with Lys\(^{16}\) (see below).

**K16Q Mutant**—The NZ atom of Lys\(^{16}\) probably interacts with the \(\beta\)- and/or \(\gamma\)-phosphate groups of GTP (5, 6). The consensus P-loop lysine is putatively essential for stabilization of a pentavalent phosphoryl group in the transition state (8) as is well documented in p21\(^{ras}\) (15) and adenylate kinase (16, 17). However, for AMPsase, the kinetic parameters of the K16Q mutant (Table I) are similar to those of the wild-type protein. The corresponding mutant of p21\(^{ras}\) (K16N) drastically reduces the affinity of nucleotides (15), and the corresponding mutant of *E. coli* adenylate kinase (K13Q) significantly lowers catalytic activity with a modest effect on substrate affinity (16). The possibility that the K16Q mutant of AMPsase may have reverted to the wild-type protein was eliminated by confirming the sequence of the mutant plasmid in the transformed H1238 cell line. Also we detected no endogenous AMPsase in the H1238 *E. coli* cell line by Western blot analysis (data not shown). Thus, apparently NE2 of glutamine can substitute for NZ of Lys\(^{16}\) in maintaining hydrogen bonds. Furthermore, for AMPsase, the positive charge of Lys\(^{16}\) may not be essential for the stabilization of the transition state. In fact, Lys\(^{16}\) hydrogen bonds to Glu\(^{14}\) in ligated complexes of AMPsase, resulting in a charge-balanced ion pair. In order for NE2 of the Gln\(^{16}\) mutant to take up the position of NZ of Lys\(^{16}\), OE1 of Gln\(^{16}\) must hydrogen bond to Glu\(^{14}\) (Fig. 3). Thus, the observed Lys\(^{16}\)-Glu\(^{14}\) ion pair in the wild-type enzyme may be replaced by a neutrally charged Gln\(^{16}\)-Glu\(^{14}\) pair. The net electrostatic charge of the mutated and wild-type active site, then, may be the same. Hence we observe little influence on kinetic parameters. Neither adenylate kinase nor p21\(^{ras}\) have a P-loop residue equivalent to Glu\(^{14}\).

If the mutation of Lys\(^{16}\) has only a modest impact on catalysis, why then is position 16 always a lysine in all known sequences of AMPsase? The explanation may rest with the stability of the mutant. At room temperature, the activity of the K16Q mutant is unchanged for 90 min, but at 4 °C the activity decreases significantly relative to that of the wild-type protein (Fig. 4). It is known that hydrophobic interactions are weakened at lower temperatures (18). In addition, the stability of AMPsase dimers decreases with temperature (19). It was also observed that when the mutant and wild-type enzymes were exposed to 0.5 M urea for varied periods of time and then assayed for activity, the K16Q enzyme was significantly less stable than its wild-type counterpart (data not shown). Taken

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**Fig. 4.** Time course for the inactivation of the K16Q mutant at 4 °C: a comparison of the stabilities of the wild-type (○) and the K16Q (□) AMPsases. The arrow indicates the starting point of the incubation at 4 °C.

**Fig. 5.** Stereo view of Arg\(^{131}\) in relation to the loop 298–300 in the crystal structure of ligated AMPsase. Hadacin, an analog and competitive inhibitor of aspartate, is drawn with **bold lines.**
together, these results suggest that the K16Q mutant is less stable than the wild-type protein, provided that one accepts enzyme activity as a criterion of stability. If minor structural alterations in the P-loop lessen the stability of AMPSase, as revealed by subunit complementation experiments (19), glutamine may not be permitted to position 16 in E. coli AMPSase due to selective pressures of evolution. However, if one argues that the conditions described in Fig. 4 and with 0.5 mM urea may not be applicable in vivo, it becomes difficult to explain the evolutionary preference for lysine over glutamine at position 16.

The conservation of lysine at positions equivalent to 16 in all known sequences of AMPSase may stem alternatively from survival disadvantages associated with a 3-fold reduction in $k_{\text{cat}}$. The wild-type activity of AMPSase in E. coli may barely meet the requirement of the cell for adenine nucleotides. Thus, even a 3-fold reduction in activity of the K16Q mutant relative to the wild-type enzyme could be catastrophic. In an attempt to evaluate the amount of AMPSase in E. coli (TG 1 cells), a quantitative Western blot experiment was employed with antibody against E. coli AMPSase. The signal from a crude extract of TG 1 cells was compared with that of purified AMPSase; the amount of AMPSase was approximately 1.5 mg of AMPSase/g of wet TG 1 cells (data not shown). Assuming that the weight percents of total DNA and RNA in E. coli are 1 and 6% (20), respectively, with a quarter of this nucleotide pool in the weight percents of total DNA and RNA in E. coli, and that the free adenine and that the free adenine $6\% (20)$, respectively, with a quarter of this nucleotide pool in the weight percents of total DNA and RNA in E. coli, and that the free adenine.

The synthetase may negate this suggestion. Based upon the amount of AMPSase in E. coli and its low turnover number, it is tempting to suggest that the AMPSase reaction is the rate-limiting factor in the generation time of the cell containing wild-type AMPSase. Such a mutation in a critical enzyme with an extremely low turnover number may not be acceptable for cell survival in a competitive environment.

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**H41N Mutant**—The H41N mutant has broadly altered kinetic constants relative to wild-type AMPSase, including a $k_{\text{cat}}$ that is approximately 0.01/s (1% of the wild-type enzyme) and $K_m$ substrate values increased by 2–6-fold relative to those of the wild-type enzyme (Table I). The uniform increase in $K_m$ values for all substrates implies that the mutation perturbs the entire active site. A hydrogen bond between His41 and the $\beta$-and/or $\gamma$-phosphoryl groups of GTP is one of three enzyme-ligand interactions putatively responsible for the 9-Å conformational change in the loop 42–53 (5). The loop 42–53 folds over the guanine nucleotide; the 6-fold increase in $K_m$ for GTP may stem from a weakened interaction between Asn41 and the guanine nucleotide relative to observed His41-guanine nucleotide interactions in the wild-type system. Increases in $K_m$ for IMP and aspartate to some extent may stem from the decrease in GTP affinity. Synergism in the binding of IMP and GTP is suggested by studies of Wang et al. (21). Furthermore, Mg$^{2+}$ binds to guanine nucleotides and putatively to the $\alpha$-carboxylate of aspartate (5). Thus, the observed 6-fold increase in the $K_m$ for aspartate may be due entirely to the 6-fold increase in the $K_m$ for GTP and, presumably, bound Mg$^{2+}$. The altered CD spectrum of the H41N mutant may not be relevant to its kinetic properties; the CD spectrum measures the conformation of the unligated mutant, whereas the kinetics probe the ligated mutant. The severely depressed $k_{\text{cat}}$ of the H41N mutant is in harmony with crystallographic studies that implicate His41 as a catalytic acid in the phosphotransfer step.

**R131L Mutant**—The guanidinium group of Arg131 is close to the loop that binds to the $\beta$-carboxylate group of aspartate (Fig. 5), and its removal affects the affinity for aspartate ($K_m$; Arg increases 4-fold) without a significant influence on other parameters. Mutation of Arg305 or Arg305 to leucine increases $K_m$ for aspartate by 100-fold (21); these residues putatively bind directly to the $\beta$- and $\alpha$-carboxylates, respectively, of aspartate. Thus, the 4-fold increase in $K_m$ of the R131L mutant is consistent with long range, electrostatic interactions between Arg131 and aspartate, and the stabilization of the aspartate-bound conformation of the loop 298–303.

**Summary**—Mutations at positions 13, 14, 41, and 131 are consistent, as noted above, with their putative roles in catalysis and conformational changes in AMPSase in E. coli. The high activity of K16Q was not anticipated on the basis of recent crystallographic structures of AMPSase, which clearly show Lys16 making hydrogen bonds with a nitrate anion in the $\gamma$-phosphoryl site (5) and with a $P_i$ anion in the $\gamma$-phosphoryl site and the $\beta$-phosphate group of bound GDP (6). On the basis of crystallographic structures, the role played by Lys16 appears to be as significant as Arg305, His41, or Asp13, where mutations cause at least a 99% reduction in $k_{\text{cat}}$. Thus, the positive charge on Lys16 does not play a significant role in stabilizing the transition state. Further mutations at position 16 should reveal whether glutamine is the next best alternative to lysine or whether other substitutions at position 16 result in mutants with significant catalytic capacity.

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³ B. W. Poland, R. B. Honzatko, and H. J. Fromm, unpublished observations.
