A Study of Escherichia coli Adenylosuccinate Synthetase Association States and the Interface Residues of the Homodimer*

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The state of aggregation of adenylosuccinate synthetase from Escherichia coli is a point of controversy, with crystal structures indicating a dimer and some solution studies indicating a monomer. Crystal structures implicate Arg143 and Asp231 in stabilizing the dimer, with Arg143 interacting directly with bound IMP of the 2-fold related subunit. Residue Arg143 was changed to Lys and Leu, and residue Asp231 was changed to Ala. Matrix-assisted laser desorption ionization mass spectroscopy and analytical ultracentrifugation of the wild-type and the mutant enzymes indicate a mixture of monomers and dimers, with a majority of the enzyme in the monomeric state. In the presence of active site ligands, the wild-type enzyme exists almost exclusively as a dimer, whereas the mutant enzymes show only slightly decreased dissociation constants for the dimerization. Initial rate kinetic studies of the wild-type and mutant enzymes show similar kcat and Km values for aspartate. However, increases in the Km values of GTP and IMP are observed for the mutant. Changes in dissociation constants for IMP are comparable with changes in Km values. Our results suggest that IMP binding induces enzyme dimerization, and that two residues in the interface region, Arg143 and Asp231, play significant roles in IMP and GTP binding.

Adenylosuccinate synthetase (AMPSase) IMP·L-aspartate ligase (GDP forming), EC 6.3.4.4. catalyzes the first committed step in the conversion of IMP to AMP in the de novo synthetic pathway for purine nucleotides:

\[ \text{Mg}^{2+} + \text{IMP} + \text{L-aspartate} + \text{GTP} \rightarrow \text{adenylosuccinate} + \text{GDP} + \text{P}, \]

\[ \text{REACTION 1} \]

Adenylosuccinate is then cleaved by adenylosuccinate lyase to form AMP and fumarate. AMPSase also plays a role in the salvage and nucleotide interconversion pathways (1). The enzyme is potentially a target of a natural herbicide (2) and of a drug used in the treatment of pediatric leukemia (3). Better understanding of the synthetase could support efforts in the design of drugs against human immunodeficiency virus and cancer (4).

Three different mechanisms have been proposed for the catalytic action of AMPSase (1). The most widely accepted mechanism involves a 6-phosphoryl-IMP intermediate formed by the nucleophilic attack of the 6-oxo group of IMP on the γ-phosphorus atom of GTP (5, 6). A second nucleophilic attack by the amino group of aspartate on C-6 of 6-phosphoryl IMP displaces the phosphate and forms adenylosuccinate.

AMPSase was first purified to homogeneity in 1976 from Escherichia coli (7) and has since been purified and characterized from many sources (1). The purA gene, which in E. coli codes for AMPSase, was cloned in 1986 (8) and used in the construction of an overexpression system (9). The crystal structure of AMPSase was determined to 2.8 Å (10) and later refined to 2.5 and 2.0 Å (11). In crystal structures, the enzyme exists as a homodimer. Two nearly independent regions contribute to the interface between the polypeptide chains of the synthetase dimer. Residues putatively involved in the binding of IMP lie at or near the interface between polypeptide chains in the dimeric form of the enzyme. One of the residues, Asp231, may play an important role in holding the subunits in close contact by hydrogen bonding to Arg143 and Lys140 of the 2-fold related subunit. Indeed, when Lys140 and Arg143 were replaced by isoleucine and leucine, respectively, Km(GTP) and Km(IMP) showed significant increases (12, 13).

Crystal structures imply that Arg143 is involved in IMP binding to the active site of the symmetry-related subunit of the dimer. Arg143 may also stabilize the interface through at least one hydrogen bond. Slow phase inactivation of AMPSase was observed when guanosine-5′-O-[S-(4-bromo-2,3-dioxobutyl)] thiophosphate modifies the enzyme at Arg143. Modification of Arg143 is prevented by adenylosuccinate alone or by a mixture of GTP, MgCl2, and IMP (14).

The cited findings suggest a direct relationship between dimer formation and AMPSase activity. AMPSase exists as a monomer in solution (15), yet the enzyme is a dimer in crystal structures (10, 11). The discrepancy between the level of aggregation of the enzyme in solution and the crystal is the basis of an important question: Does AMPSase function as a monomer or as a dimer? MALDI mass spectroscopy, analytical ultracentrifugation, and initial rate kinetics were used to determine the state of aggregation and activity of wild-type AMPSase and several interface mutants. Arg143 was replaced with leucine to remove the guanidinium group and yet retain some of the hydrophobic attributes of the original side chain. Mutation of Arg143 to lysine retains the positive charge but limits hydrogen bonding opportunities of the side chain at position 143. Asp231 was replaced with alanine so as to disrupt the Lys140-Asp231 salt link observed in the crystalline dimer.

Our findings indicate that the E. coli enzyme dimerizes in response to active site ligands and that on the basis of sequence homology, dimerization may be a property common to all known adenylosuccinate synthetases, regardless of source.

*The abbreviations used are: AMPSase, adenylosuccinate synthetase; MALDI, matrix-assisted laser desorption ionization.
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EXPERIMENTAL PROCEDURES

Materials—GTP, IMP, l-aspartate, phenylmethylsulfonyl fluoride, and bovine serum albumin were obtained from Sigma. A site-directed mutagenesis kit was obtained from Amersham Corp. Restriction enzymes were obtained from Promega. E. coli strain XL-1-blue was obtained from Stratagene. E. coli strain purA-H1238 was a gift from Dr. D. Bachman (Genetic Center, Yale University). Other reagents and chemicals used in the experiments were obtained from Sigma if not specified.

Site-directed Mutagenesis—Recombinant DNA manipulation was performed by using standard procedures (16). The plasmid containing a 1.8-kilobase BamHI-HindIII fragment from PMS204 ligated into PUC118 was used in the mutagenesis step. All mutagenic oligonucleotide primers used in the experiments were synthesized on a Biosearch 8570EX automated DNA synthesizer at the DNA Facility at Iowa State University. Mutagenesis was performed according to the protocol provided by Amersham Corp. The mutations were confirmed by DNA sequencing using the chain termination method (17) at the Iowa State University DNA Facility. The 1.8-kilobase Bam HI-HindIII fragment with the desired mutation was ligated back into PMS204 and transformed into XL-1 blue cells. The plasmids isolated from that strain were used to transform E. coli strain purA-H1238, the strain from which AMPase was purified.

Protein Assay—Protein concentration was determined by the Bradford (18) method using bovine serum albumin as the standard. Concentrations reported here refer to monomers.

Kinetic Studies of Wild-type and Mutant Adenylosuccinate Synthetase—The wild-type and mutant enzymes were purified as described elsewhere (9). The purity of the enzyme was checked by SDS-polyacrylamide gel electrophoresis according to Laemmli (19). AMPase activity was determined as described earlier (20).

Kinetic Studies of Wild-type and Mutant AMPase—The concentrations of stocks solutions of nucleotides were based on their molar extinction coefficients at 253 nm for GTP and 248 nm for IMP. For each enzyme assay, the increase at 290 nm was recorded at 25 °C. The enzyme assay solution contained 20 mM Hepes (pH 7.7) and 5 mM MgCl2. When GTP was the variable substrate, the concentration of aspartate was fixed at 5 mM, and the IMP concentration was fixed at 450 μM for wild-type AMPase and at 12 mM for the mutants. When IMP was the variable substrate, the concentration of GTP was fixed at 300 μM, and aspartate was fixed at 5 mM. When aspartate was the variable substrate, the concentration of GTP was fixed at 300 μM, and the concentration of IMP at 450 μM for the wild-type AMPase and 12 mM for the mutants.

To obtain the values of KΙΜ and KΙα, dissociation constants for GTP and IMP, respectively, a 5 × 5 matrix of substrates were used with each enzyme. The enzyme assay solution contained 20 mM Hepes (pH 7.7), 5 mM MgCl2, and 5 mM aspartate and varying GTP and IMP concentrations. The kinetic data were fit to Equation 1 using a program written in Minitab in place of the Omnitab program described by Siano (21):

\[
\frac{1}{v} = \frac{1}{V_m} + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{a,b}}{AB}
\]

where \( v \) is the initial rate, \( V_m \) is the maximal velocity, \( A \) is the GTP concentration, \( B \) is the IMP concentration, \( K_a \) is the Michaelis constant for GTP, \( K_b \) is the Michaelis constant for IMP, and \( K_{a,b} \) is the dissociation constant for GTP and IMP, the dissociation constant for IMP.

Circular Dichroism Spectroscopy—Circular dichroism spectra for the wild-type and mutant enzymes were acquired on a JASCO J700 spectropolarimeter equipped with a data processor. Samples (100 μg/ml of enzyme) dialyzed against 5 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM β-mercaptoethanol, were placed in a 1-cm cuvette, and data points were collected in 0.1-nm increments. Each spectrum was corrected for background contributions of the buffer and smoothed using the spectropolarimeter program. The data were analyzed by JASCO software or by PSIPLOT.

MALDI Mass Spectroscopy—All of the enzymes were dialyzed against 5 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, and 1 mM β-mercaptoethanol. Protein concentration was adjusted to 1 mg/ml. Samples of 0.5–1.0 μl were loaded with 0.5 μl of freshly made 3.5-dimethoxy-4-hydroxy-cinnamic acid matrix. Bovine serum albumin was used as the internal calibration standard. Data were collected on a Finnigan LASERMAT 2000 MALDI-time of flight mass analyzer by the Protein Facility of Iowa State University and were analyzed by the LASERMAT 2000 data processing software.

Analytical Ultracentrifugation (Sedimentation Equilibrium) of Wild-type and Mutant AMPase—Analytical ultracentrifugation experiments were performed using a Beckman Optima XL-A ultracentrifuge. The temperature of the rotor (AN-50 Ti) was set at 4 °C. Rotor speeds were set at 10,000, 14,000, and 18,000. Wild-type and mutant AMPase samples were prepared in 5 mM potassium phosphate buffer (pH 7.0) at concentrations of 2.9–11.6 μM corresponding to absorbances of 0.2–0.8 (280 nm, 1-cm cuvette). Concentration-dependent equilibrium sedimentation was performed with concentrations of 4.3 and 58 μM, corresponding to A280 readings of 0.3 and 4.0. Samples in the presence of ligands were prepared by dialysis overnight against 5 mM potassium phosphate (pH 7.0), 5 mM succinate, 5 mM MgCl2, 20 μM IMP, and 20 μM GTP. Protein samples were centrifuged at least 10 h before the collection of data. Stepwise radial scans were performed at 280 nm for the wild-type and mutant enzymes with and without ligands, and at 280, 285, and 300 nm for the concentration-dependent equilibrium sedimentation. Each reading is the average of 30 points with nominal spacing of 0.001 cm between radial positions. Absorbance readings were measured at 1-h intervals to ensure that equilibrium had been reached. Three scans were averaged, and the data were analyzed by the method of Van Holde and Weischat (22) using the “SELF” model in multiple data set analysis program Optima XLA. The association constants (Kd) for the monomer-dimer equilibrium were obtained using the following equation:

\[
c = E + c_{m,exp}[1 + \gamma\mu_0\sqrt{r^2 - \rho^2}/2\pi R] + (c_{m,exp})^2 K_{exp}
\]

\[
[2M(1 - \gamma\mu_0\sqrt{r^2 - \rho^2}/2\pi R)]
\]

where c is the concentration of the protein at a given radial position, \( c_{m,exp} \) is the concentration of monomer at a reference position, M is the monomer molecular mass, \( \mu_0 \) is the partial specific volume, \( \rho \) is the solvent density, \( \omega \) is the angular velocity, \( r \) is the radial position in centimeters from the center of rotation, \( r_s \) is the distance in centimeters from the center of rotation to the meniscus, \( T \) is the absolute temperature, \( R \) is the gas constant, and E is a correction term for a nonzero baseline. The conversion of Kd into the dissociation constant Kd(μM) is performed as follows:

\[
K_d = (2eK_d) = 24.2/K_{d(μM)}
\]

RESULTS

X-ray crystallographic studies suggest that interface residues play an important role in maintaining the quaternary structures of AMPase (10, 11). Two very important residues in this context are Arg143 and Asp231. Arg143 in one subunit is ligated to the 5′-phosphoryl of IMP in the active site of the juxtaposed subunit (11). Asp231 forms a salt link with Lys140, a residue putatively essential for AMPase activity as a means of stabilizing subunit-subunit association. Experiments involving mutation of residues Arg143 and Asp231 were undertaken to gain insight into the role of these interface residues.

Comparison of the Sequences 138–148 and 228–235 in E. coli AMPase with the Synthetases from Other Sources—Arg143 and Asp231 are conserved in the nine known AMPase sequences (8, 24–29, 31) (Table I), as are Arg147 and Lys140. In crystal structures, the side chain of Asp231 forms a salt bridge with Lys140, and the carbonyl of Asp231 hydrogen bonds to Arg147 (10, 11). Chemical modification or mutation of Lys140 or Arg147 inactivates the synthetase (12, 13). Arg143 from each sequence (8, 24–29, 31) (Table I), as are Arg147 and Lys140. In crystal structures, the side chain of Asp231 forms a salt bridge with Lys140, and the carbonyl of Asp231 hydrogen bonds to Arg147 (10, 11). Chemical modification or mutation of Lys140 or Arg147 inactivates the synthetase (12, 13). Arg143 from each

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2 F. M. Tatum and M. A. Steckelberg, unpublished data.
cation of the Mutant Enzymes—The oligonucleotide primers used in the mutagenesis experiments are shown in Table II together with the sequencing primers for confirming the mutants. Arg143 was changed to leucine and lysine. Asp231 was mutated to alanine. These mutations altered the charge states of residues and/or hydrogen bonding interactions observed in crystallographic structures while keeping the size of the side chain comparable with that of the original residue. All the mutants were purified by using procedures similar to those for wild-type AMPSase with some modifications. The D231A mutant bound to the phenyl sepharose CL-4B so tightly that it could be eluted only by water. All the enzymes exhibited greater than 95% purity on the basis of SDS-polyacrylamide gel electrophoresis and a molecular mass of 48 kDa (data not shown).

Secondary Structure Analysis—Circular dichroism spectra of the mutant and wild-type enzymes were superimposable (data not shown) from 200 to 260 nm, indicating no global alterations in the secondary structures of the mutants relative to wild-type AMPSase.

Kinetic Analysis of AMPSase Mutants—The kinetic parameters for GTP, IMP, and aspartate with various forms of AMPSase are summarized in Table III. The $K_m$ values for aspartate illustrate that the mutants differed little from that of the wild-type enzyme, suggesting that Arg143 and Asp231 are not involved in the binding of aspartate. In addition, the mutant and wild-type AMPSases had comparable $k_{cat}$ values; however, $K_m$ values for GTP and IMP exhibited significant increases for the mutant enzymes relative to wild-type AMPSase. The $K_m^{(GTP)}$ values for R143K, R143L, and D231A showed 2-, 10-, and 20-fold increases, respectively, compared with that of the wild-type enzyme. On the other hand, even more dramatic changes were observed for the $K_m$ values of IMP. Increases in $K_m^{(IMP)}$ of 100-, 100-, and 60-fold for the mutants R143K, R143L, and D231A, respectively, were observed relative to that of wild-type AMPSase. In addition, increases in $K_m^{(IMP)}$ of 20–30-fold were also observed for the mutant enzymes, relative to that of the wild-type enzyme. These findings suggest that the residues in question contribute significantly to the binding of both IMP and GTP by stabilizing the dimer through hydrogen bonding, by direct interaction with IMP, or by both. The similar kinetic properties of R143K and R143L mutants with respect to IMP binding suggest that the positive charge of lysine alone did not restore wild-type properties, suggesting a precise hydrogen bonding and stereochemical role for Arg143. The models for R143K and R143L are presented and discussed below (Fig. 1).

MALDI Mass Spectrometry Analysis of AMPSase—AMPSase in solution is a monomer (15), whereas it is a dimer in crystals (10, 11). We assumed that subunit association might involve interface residues such as Arg143; therefore, experiments were undertaken to evaluate this possibility. The mutant enzymes and wild-type AMPSase were analyzed by MALDI mass spectrometry. A typical spectrum is shown in Fig. 2. Given the estimated molecular mass of the monomer as 46 kDa, three ionized species were observed from 20 to 110 kDa, corresponding to mass values of 23.4 kDa ($M = 46.6$ kDa, $Z = 2$), 46.6 kDa ($M = 46.6$ kDa, $Z = 1$), and 92.7 kDa ($2M = 92.7$ kDa, $Z = 1$). The existence of a 92.7-kDa peak indicates the presence of dimers.

Equilibrium Sedimentation of Wild-type and Mutant AMPSases—Subunit association of wild-type AMPSase was evaluated at three different concentrations (32). Considering the molecular mass of the monomeric and dimeric enzymes, three different centrifugation speeds were utilized (10,000, 14,000, and 18,000). The molecular mass of AMPSase does not depend on either the centrifugation speed or the protein concentration under the conditions tested. Typical equilibrium sedimentation data are shown in Fig. 3a. Fig. 3b shows the relationship between wild-type protein concentration and apparent molecular mass. At low concentrations ($A_{250}$ nm $= 0.1$), the apparent molecular mass was 45 kDa, which matches the result determined by MALDI mass spectroscopy. At higher concentrations ($A_{250}$ nm $= 0.65$), the apparent molecular mass approaches 60 kDa, which is between the molecular mass for monomeric and dimeric AMPSase. This observation strongly indicates the existence of a monomer-dimer equilibrium. Dissociation constants for dimer-monomer equilibria of wild-type and mutant AMPSases are shown in Table IV. At micromolar concentrations of enzyme in the absence of ligands, the $K_d$ values are approximately $10 \mu M$ for all the enzymes, indicating low concentrations of dimers under these conditions. In the presence of active site ligands, the mutant enzymes exhibited small decreases in $K_d$ values, suggesting a slight increase of dimers, whereas the wild-type enzyme exhibited a large decrease in $K_d$, implying that virtually all the protein is present as dimer. Active site ligands apparently are less effective in the stabilization of the mutant dimers. Our findings support the important roles played by the interface residues in forming AMPSase dimers.

### Table I

| Sources                | Sequences*  | References |
|------------------------|-------------|------------|
| B. subtilis            | MSEKAIKGR1  | 24         |
| D. acidocoides         | SSSKASGLVR  | 25         |
| E. coli                | EVKVRGRGLV | 8          |
| Human liver            | SEKALQRM   | 26         |
| Mouse muscle           | SEKARGlR    | 27         |
| Mouse T lymphoma cells | SEKASLRM    | 28         |
| Haemophilus Influenza  | EVKQVRLG   | 29         |
| Brucella abortus       | EIVGQARRV  | 30         |
| S. cerevisiae          | STKASLRGLV | 31         |

* The conserved residues corresponding to Lys140, Arg143, Arg147, and Asp231 in E. coli AMPSase from different sources are shown in bold type.

### Table II

| Mutants | Sequences of primers* |
|---------|-----------------------|
| R143L   | CAGACGGCCGAGATGCTCACTTTACTC |
| R143K   | CAGACCGCGTIITGCTCACTTTATTC |
| D231A   | TAAGTACGGTGAGCCTACCGAG |
| Sequencing primer for residue 143 | CTGGTGGAAGAAGATGCC |
| Sequencing primer for residue 231 | ATGGGTTGTTGCGACATC |

* Underlined letters indicate the mismatches.

### Table III

| Protein  | $k_{cat}$ | $K_m^{(GTP)}$ | $K_m^{(IMP)}$ | $K_{cat}^{(GTP)}$ | $K_{cat}^{(IMP)}$ |
|----------|-----------|---------------|---------------|--------------------|-------------------|
| Wild type| 1.00 ± 0.05 | 26.2 ± 3.20 | 0.03 ± 0.00 | 0.23 ± 0.04 | 38.8 ± 6.40 |
| R143K    | 1.04 ± 0.01 | 58.3 ± 6.30 | 2.50 ± 0.25 | 0.26 ± 0.05 | 31.0 ± 5.92 |
| R143L    | 1.01 ± 0.01 | 283 ± 6.22  | 2.79 ± 0.13 | 0.34 ± 0.06 | 196 ± 37.6  |
| D231A    | 0.76 ± 0.02 | 503 ± 41.3  | 1.31 ± 0.23 | 0.50 ± 0.03 | 197 ± 62.3  |

* Experimental conditions as described under “Experimental Procedures.”

**Underlined letters indicate the mismatches.**
DISCUSSION

Conflicting reports have characterized *E. coli* AMPSase as a monomer (15) and a dimer (9) in solution. Crystal structures of AMPSase clearly show the enzyme as a dimer in both the unligated (10, 11) and substrate-ligated states (33). AMPSase is putatively regulated by such feedback inhibitors as GDP, adenylosuccinate, and adenine nucleotides (1). The concentration of the latter class of compounds may not vary in the cell.

**FIG. 1.** a, stereo view of the AMPSase dimer complexed with IMP. b, stereo view of the interaction of IMP with Arg^{143}. c, stereo view of the interaction of IMP with modeled Lys^{143}.
because of the adenylate kinase equilibrium. An alternative mode of regulation may be linked to the state of association of the enzyme. We therefore undertook experiments to determine the enzyme’s state of association under different experimental conditions.

Protein aggregation is influenced by protein concentration, ligands, pH, temperature, and ionic strength (34–36). Discrepancies in reported molecular masses of AMPsase from different sources may stem from the precise conditions under which mass determinations were made (1). We used two widely differing approaches, MALDI mass spectroscopy and equilibrium sedimentation, to determine molecular mass. Data from MALDI mass spectroscopy clearly revealed the presence of a 92-kDa species (Fig. 2), a finding fully consistent with the existence of AMPsase as a dimer in solution. The MALDI technique, however, cannot provide a measure of the relative amounts of monomer and dimer. Equilibrium sedimentation was performed to determine whether a dynamic equilibrium exists between the monomer and dimer forms. Our results suggest that without ligands, AMPsase is a mixture of both monomers and dimers (50% monomer and 50% dimer at 11.4 mM wild-type enzyme) and the equilibrium shifting to favor the dimer at higher concentrations (91% dimer and 9% monomer at 58.2 mM wild-type enzyme). These observations are in harmony with x-ray diffraction studies in which only dimers were observed (10, 11, 33).

We failed to observe two distinct species in centrifugation experiments, suggesting that a rapid equilibrium exists relative to the sedimentation rate between the monomers and dimers in the absence of ligands. Only dimers were detected, however, when substrates were added to the wild-type enzyme. Ligands clearly shift the equilibrium toward the dimer and the kinetic barrier in dimer formation, and dissociation is relatively low. On the other hand, mutant enzymes in the presence of ligands exhibited only slightly lower $K_m$ values than in the absence of ligands, indicating a much weaker ligand-induced dimerization of mutant enzymes relative to the wild-type enzyme. Considering that Arg$^{143}$ and Asp$^{231}$ are well conserved in all the AMPsase sequences, the results here also suggest that AMPsase from *E. coli* may require both subunits for catalytic activity at physiological ligand concentrations and that this may be a general property of all AMPsases, regardless of source. AMPsase, however, is not the only protein that changes its state of subunit association upon ligand binding. Briehl demonstrated that lamprey hemoglobin exists predominantly as monomers when oxygenated and as oligomers when deoxygenated (37).

This study focused on two residues of AMPsase, Asp$^{231}$ and Arg$^{143}$, that are involved in putative subunit-subunit interactions. Arg$^{143}$ from one subunit hydrogen bonds to the 5′-phosphoryl group of IMP in the active site of the symmetry-related subunit (33). In addition, Arg$^{143}$ hydrogen bonds to a backbone carbonyl of the juxtaposed subunit. Because Arg$^{143}$ is conserved in all nine known sequences of AMPsase (8, 24–29, 31), we suggest that its role in the *E. coli* enzyme is also conserved in all other known AMPsases. The mutants R143L and R143K exhibit approximately the same $k_{cat}$ and $K_m$ values for aspartate as does wild-type AMPsase with small changes in the $K_m$ values for GTP (2- and 10-fold increases, respectively, for R143K and R143L). However, 100-fold increases in $K_m$ for IMP were observed for both mutant enzymes. Despite great differences in the hydrophobicity and electrostatic charge of lysine and leucine residues at position 143, they exhibit very similar kinetic properties in terms of IMP binding.

The spatial relationship of the side chain of position 143 to the active site of the monomer related by the 2-fold symmetry is shown in Fig. 1. The guanidinium group of Arg$^{143}$ bonds to the backbone carbonyl of the symmetry related subunit, as well as the 5′-phosphate of IMP (Ref. 33 and Fig. 1b). By model building, atom NZ of lysine 143 can occupy the same position as the guanidinium nitrogen responsible for the intersubunit hydrogen bonds (Fig. 1c). Thus, the increase in $K_m$ for the R143K mutant is not immediately obvious from the modeling study. However, the substitution of a lysyl for an arginyl side chain at position 143 leaves a packing void at the interface between monomers. Presumably this void is filled by a water molecule,
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The dissociation constant in 280 nm absorbance units.

The dissociation constant in μM determined by Equation 3 shown under “Experimental Procedures.”

Goodness of fit was determined by the χ² test (32).

In the absence of ligands

| Enzyme | A_{280\,nm} | K_{a} | K_{b} | Goodness of fit μM |
|--------|-------------|-------|-------|-------------------|
| Wild type | 0.3 | 0.464 | 11.2 | 0.030 |
| R143K | 4.0 | 0.170 | 4.12 | 3.54 |
| R143L | 0.3 | 0.452 | 10.9 | 0.777 |
| D231A | 0.2 | 0.384 | 9.53 | 0.849 |
| D231A | 0.3 | 0.362 | 8.76 | 0.207 |

In the presence of ligands

| Enzyme | A_{280\,nm} | K_{a} | K_{b} | Goodness of fit μM |
|--------|-------------|-------|-------|-------------------|
| Wild type | 0.4 | 4.26 \times 10^{-40} | 1.03 \times 10^{-38} | 0.00019 |
| R143K | 0.4 | 0.179 | 4.21 | 1.96 |
| R143L | 0.4 | 0.307 | 7.43 | 2.07 |
| D231A | 0.4 | 0.208 | 5.03 | 0.151 |

Synthesis is functionally active (11). The increase in $K_m$ for GTP with the mutants may be due to synergism in the binding of IMP and GTP. Markham and Reed, for instance, observed synergism in the inhibition of AMPSase by GDP and nitrate (38, 39), both of which are competitive inhibitors with respect to GTP. Furthermore, on the basis of isotopic scrambling reactions (40), the γ-phosphoryl group of GTP probably exchanges between the two sites, where it is either covalently linked to the 6-oxo group of IMP or covalently linked to the β-phosphoryl group of the guanine nucleotide. In the absence of the dimer interface, AMPSase cannot provide an appropriate environment for binding IMP, which probably impairs the exchange process just described and leads to a weaker association of GTP with the enzyme.

Combining the two lines of evidence from biophysical analysis and initial rate kinetics on both the wild-type and mutant enzymes, we established a clearer relationship between the association states and enzymatic activity of E. coli AMPSase. MALDI mass spectroscopy revealed the existence of a 92-kDa species for all the mutant enzymes, indicating that mutations at positions 231 and 143 do not prevent dimerization. R143L, in the presence of ligands, showed an apparent molecular mass of a dimer as determined by gel filtration analysis (data not shown). These observations suggest that neither Arg143 nor Asp231 play a role in the rate-limiting step of catalysis (interconversion from AMPSase-MgGTP-2-IMP-aspartate to AMPSase-adenylosuccinate-MgGDP-1-P)

An interesting question concerns the significance of the AMPSase monomer-dimer equilibrium and its physiological implications, if any. The enzyme should exist primarily as a dimer in the micromolar concentration range, and substrates should shift the monomer-dimer equilibrium to the dimer. Only if the monomer and dimer have dissimilar activities at physiological ligand concentrations can the monomer-dimer equilibrium be of significance to regulation. Interestingly, in the enzyme concentration range of 20–150 μM, there is a several minute lag in adenylosuccinate production when the reaction is initiated with the enzyme (data not shown). When the
enzyme is preincubated with MgGTP<sup>−2</sup> and IMP and the reaction is initiated with L-aspartate, however, the lag is eliminated. These findings are consistent with either an inactive monomer or a monomer of low activity relative to the dimer. Perhaps AMPSase is a monomer in the absence of nucleotide substrates but is activated by de novo purine nucleotide biosynthesis. This scenario may represent a major control mechanism of adenylosuccinate biosynthesis.

It is also noteworthy that AMPSase from Saccharomyces cerevisiae was identified as a single stranded DNA binding protein that specifically recognizes an autonomously replicating sequence (30). Because most of the single stranded DNA-binding proteins are oligomers, the dimerization phenomenon may indicate a dual role for AMPSase as an enzyme and as a regulatory element in DNA replication.

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REFERENCES

1. Stayton, M. M., Rudolph, F. B., and Fromm, H. J. (1983) Curr. Top. Cell Regul. 22, 103–141.
2. Heim, D. R., Cseke, C., Gewick, B. C., Murdoch, M. G., and Green, S. B. (1995) Pestic. Biochem. Physiol. 53, 138–145.
3. DeAbreu, R. A. (1995) Purine and Pyrimidine Metabolism in Man VIII, pp. 195–200, Plenum Press, New York.
4. Ahluwalia, G., Cooney, D. A., Mitsuya, H., Fridland, A., Flora, K. P., Hao, Z., Laemmli, I. (1956).
5. Poland, B. W., Silva, M. M., Serra, M. A., Cho, Y., Kim, K. H., Harris, E. M. S., Rudolph, F. B., and Fromm, H. J. (1969) J. Biol. Chem. 244, 3832–3839.
6. Stayton, M. M., and Fromm, H. J. (1977) Arch. Biochem. Biophys. 170, 587–600.
7. Wolfe, S. A., and Smith, J. M. (1988).
8. Bradfrod, M. M. (1976) J. Biol. Chem. 251, 49–54.
9. McRorie, D. K., and Voelker, P. J. (1993).
10. Poland, B. W., Silva, M. M., Hoffman, C. R., Fromm, H. J., and Honzatko, R. B. (1993) Arch. Biochem. Biophys. 293, 347–357.
11. Silva, M. M., Poland, B. W., Hoffman, C. R., and Honzatko, R. B. (1995) J. Biol. Chem. 270, 30593–30597.
12. Dong, Q., and Fromm, H. J. (1991) J. Biol. Chem. 266, 23854–23859.
13. Attri, A. K., Lewis, M. S., and Korn, E. D. (1991) J. Biol. Chem. 266, 22988–22993.
14. Li, Y., and Willard, J. (1990) J. Biol. Chem. 265, 6225–6230.
15. Markham, G. D., and Reed, G. H. (1975) FEBS Lett. 32, 68–72.
16. Rudolph, F. B. (1971) J. Biol. Chem. 246, 2889–2896.
17. Rudolph, F. B., and Fromm, H. J. (1969) J. Biol. Chem. 244, 3832–3839.
18. Rudolph, F. B. (1971) J. Biol. Chem. 246, 2889–2896.
19. Rudolph, F. B. (1971) J. Biol. Chem. 246, 2889–2896.
20. Rudolph, F. B., and Fromm, H. J. (1969) J. Biol. Chem. 244, 3832–3839.
21. Siano, D. B., Zyskind, J. W., and Fromm, H. J. (1975) Arch. Biochem. Biophys. 170, 587–600.
22. Van Holde, K. E., and Weisheit, W. O. (1978) Biopolymers 17, 1387–1403.
23. Cohn, E. J., and Edsall, J. T. (1943) Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, pp. 370–381, Reinhold, New York.
24. Mantesla, P., and Zalkin, H. (1992) J. Bacteriol. 174, 1883–1890.
25. Wissmuller, L., Witthodt, J., Noegel, A. A., and Schleicher, M. (1991) J. Biol. Chem. 266, 2480–2485.
26. Powell, S. M., Zalkin, H., and Dixon, J. E. (1992) FEBS Lett. 303, 4–10.
27. Guicherit, O. M., Cooper, B. F., and Fromm, H. J. (1989) J. Biol. Chem. 264, 22582–22587.
28. Guicherit, O. M., Cooper, B. F., Rudolph, F. B., and Kellems, R. E. (1994) J. Biol. Chem. 269, 4488–4496.
29. Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., Merrick, J. M., McKenney, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J. D., Scott, J., Shiehui, T., Liu, L., Glodek, A., Kelley, J. M., Weidman, J. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghegan, N. S. M., Gnehm, C. L., McDonald, L. A., Stanil, K. V., Fraser, C. M., Smith, H. O., and Venter, J. C. (1995) Science 269, 496–512.
30. Zeidler, R., Hobert, O., Johannes, L., Faulhammer, H., and Krauss, G. (1993) J. Biol. Chem. 268, 20191–20197.
31. Andreichuk, I. V., Schubes, A. V., Ryzhova, T. A., Kotova, I. A., and Domnik, V. D. (1995) Mol. Gen. Microbiol. Virusol. 1, 21–28.
32. McRorie, D. K., and Voelker, P. J. (1993) Self-Associating Systems in the Analytical Ultracentrifuge, p. 8, Beckman, Fullerton, CA.
33. Poland, B. P., Fromm, H. J., and Honzatko, R. B. (1996) J. Mol. Biol., in press.
34. Malinowski, D. P., and Fridovich, I. (1979) Biochemistry 18, 5055–5060.
35. Atti, A. K., Lewis, M. S., and Korn, E. D. (1991) J. Biol. Chem. 266, 6815–6824.
36. Lewis, M. S., and Youle R. J. (1986) J. Biol. Chem. 261, 11571–11577.
37. Brien, R. W. (1963) J. Biol. Chem. 238, 2361–2366.