Studies on the structure-function relationships and regulation mechanisms of Escherichia coli adenylosuccinate synthetase

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Studies on the structure-function relationships and regulation mechanisms of *Escherichia coli* adenylosuccinate synthetase

by

Wenyan Wang

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

Wenyan Wang

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

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For the Major Program

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For the Graduate College
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LIST OF NOMENCLATURE

AMPSase: adenylosuccinate synthetase
AMPS Lyase: adenylosuccinate lyase
AMPS: adenylosuccinate
Ala (A): alanin
ARS: autonomous replicating sequence
Asp (D): aspartate
Arg (R): arginine
AMP: adenosine phosphate
AMPS: adenylosuccinate
ADP: adenosine diphosphate
ATP: adenosine triphosphate
CD: circular dichroism
E. coli: Escheichia coli
EF-Tu: elongation factor Tu
Glu (E): glutamate
Gln (Q): glutamine
GTP: guanosine triphosphate
HPLC: high-performance liquid chromatography
IMP: inosine monophosphate
K_m: Michealis constant
kDa: kilodalton

KPi: potassium phosphate

Lys (K): lysine

MALDI: matrix assisted liquil desorption/ionization

Met (M): methionine

Pi: orthophosphate

pI: isoelectric point

SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

ssDNA: single stranded DNA
GENERAL INTRODUCTION

The de novo synthesis of AMP and GMP are connected by the common precursor IMP. Genes for the 14 steps de novo purine nucleotide synthesis have been cloned from Bacillus subtilis.(1). With the availability of large amount of recombinant enzymes, it is of great interest to decode their molecular mechanisms to shed light on potential chemotherapeutic drug design (2-5).

AMPSase [IMP: L-aspartate ligase (GDP-forming), E.C. 6.3.4.4] is a ubiquitous enzyme catalyzing the first committed step from IMP to AMP, and has been subjected to extensive studies in the past 40 years (6) The enzyme converts IMP and aspartate to adenylosuccinate (AMPS), coupling the hydrolysis of GTP in the existence of Mg\(^{2+}\).

\[
\text{IMP} + \text{GTP} + \text{aspartate} \xrightarrow{\text{Mg}^{2+}} \text{adenylosuccinate} + \text{GDP} + \text{Pi}
\]

AMPS is then cleaved by AMPS lyase to produce AMP with the release of fumarate. The enzyme is also involved in the salvage and nucleotide interconversion pathway.

Comparison of AMPSase sequences from different sources indicated 50-90% sequence identity, suggesting a well conserved biological function of this enzyme in the living systems throughout evolution. Despite some variations, two common consensus sequences for GTP recognition were observed by aligning AMPSase with GTP-binding proteins. One is the “P-loop” sequence which is supposed to recognize the phosphate moiety of GTP. The other element is the “N/TKXD” sequence of which the Asp residue
plays a critical role in GTP binding and enzyme catalysis. These findings were later supported by X-ray crystallographic studies and site-directed mutagenesis (7-10).

Studies of purified AMPSase from different biological sources indicated the existence of two distinctly different isozymes, designated as type L (pI = 8.9) and type M (pI = 5.9) (6). Based on their unique inhibition patterns, it was proposed that the acidic isozyme is primarily involved in de novo purine nucleotide biosynthesis, whereas the basic isozyme is associated with the purine nucleotide cycle. The E. coli AMPSase, either recombinant or natural, was found to be an acidic enzyme with a molecular mass of 56 kDa based on gel filtration (11), corresponding to the monomer. Activity is reported under the regulation by substrate availability, feedback inhibition, stringent response, and transcriptional regulation by purR repressor using ATP as the corepressor (6). Recent crystalline structure indicated that the enzyme is a homodimer, with a two-fold molecular symmetry (12,13). It raised interesting questions about this unique three-substrate enzyme system: Do both monomers and dimers coexist? And if the answer is yes, how is their relationship correlated with the enzyme function? What is the physiological significance of the monomer - dimer equilibrium? In the crystal structure of the ligated enzyme (Poland and Honzatko, unpublished observations), Arg143 is located on the dimer interface, with the guanidinium group forming a hydrogen bond with the 5' phosphoryl group of IMP. Site directed mutagenesis was conducted to make the mutations which potentially should disrupt subunit - subunit interactions. Initial rate kinetics indicated that residues Arg143 and Asp231 play important roles in IMP recognition, with a possible synergistic effect on GTP binding as well. MALDI mass spectroscopy and analytical ultracentrifugation were utilized to analyze
the wild type and mutant enzymes R143K, R143L, and D231A, with or without the active site ligands. The concentration of ligands at which wild-type AMPSase exhibits almost 100% dimer failed to produce a similar aggregation effect for any of the three mutant enzymes. In addition, a lag phase in the kinetic assay profile was observed when wild type AMPSase in picomolar range of was used to start the reaction with saturating levels of substrates, whereas preincubation of a similar amount of enzyme with IMP, completely eliminated the hysteresis phenomenon. For the first time, our data unambiguously suggested the ligand-induced dimerization as a regulation mechanism for AMPSase activity. Considering the homostasis of adenylate and guanylate, and the fluctuation in IMP concentrations in response to biological conditions, the rapid equilibrium between the two enzyme forms may serve as the major controlling step in de novo AMP synthesis in all organisms. It is also possible that dimerization of AMPSase may relate to other biological functions. AMPSase from Saccharamyces cerevisiae was identified as a ssDNA binding protein recognizing the ARS element, while basic AMPSase from muscle was found to be associated with F-actin. Since most ssDNA binding proteins function in their symmetrically oligomeric form, active AMPSase may provide more AMP through the purine nucleotide cycle in muscle, and dimerization of AMPSase in response to a sudden increase in the IMP supply may serve roles in DNA replication and energenesis. Further experiments using DNA footprinting, gel retardation assay, along with protein-protein interaction studies should be able to provide insights into some of these issues.

As an enzyme at a regulatory point in purine nucleotide metabolism, AMPSase has specific substrate binding sites. GTP can be substituted by dGTP or GTPyS without
compromising much of the activity (14, 15). Hydroxylamine is an only discovered alternative substrate for aspartate, although the coopertativity for Mg$^+$ is lost in the latter case (This thesis work). Reduced activity was observed when one of the phosphoryl oxygens of IMP was substituted by N, S or C atoms (16). Interestingly, IMP analogs 8-aza-IMP, ara-AMP, and allopurinol ribonucleotide are substrates for AMPSases from Leishmania donovani and Trypanosoma cruzi, but not the enzyme from mammalian sources (6). Allopurinol ribonucleotides, acting as antileishmanial agents, can be specifically incorporated into RNA after converted to 4-aminopyrazolo(3,4-d)-pyridine by protozoan AMPSase and AMPS lyase (17). Similar reactions are not catalyzed by the mammalian enzyme. These findings indicated the possible variations in the enzyme active sites despite the conservation of AMPSase function.

Attempts were made to determine the identity of the amino acid residues involved in the substrates recognition and catalysis mechanisms. A poorly ordered loop from residue 298 to 304 is located near the GTP binding site, with Arg305 associated with strong electron density, approaching the phosphate moiety of GTP (12). Three arginine residues, Arg303, Arg304, and Arg305 were replaced by leucine, to examine their functional roles. Initial rate kinetics analysis indicated that they are critical determinants in binding substrate L-aspartate (18). The finding was further supported by successful co-crystalyzation of AMPSase with hadacidin (N-formyl-N-hydroxyaminoacetic acid), a competitive inhibitor of aspartate.

Using [6-$^{18}$O]IMP, Lieberman suggested that the AMPSase reaction involves the formation of the intermediate, 6-phosphorl IMP (21). Kinetic studies by Rudolph and
Fromm suggested that the kinetic mechanism is rapid equilibrium random ter ter (11). In this mechanism, the reaction does not begin until all substrates bind to the enzyme. However, isotope exchange studies at chemical equilibrium imply that aspartate prefers add to the enzyme after the addition of the nucleotide substrates rather than to the free enzyme (21). The suggestion of Lieberman (19) that the γ-phosphoryl group of GTP is transferred to 6-oxo position to form the intermediate product 6-phosphoryl IMP plus GDP Mg\(^{++}\), is supported by isotope scrambling experiments (21). Computer modeling indicated that Gln224 is located in the vicinity of the 6-oxo of IMP and phosphates of GTP. Belonging to an highly disordered loop, it may be subjected to conformational changes upon binding of the ligands. The amide hydrogen atoms may form hydrogen bonds with 6-oxo and N-7 of IMP. Site directed mutagenesis was carried out to elucidate the roles of Gln224. Two mutants Q224E and Q224M were constructed and kinetic studies were utilized to understand its functions. Our data suggested that under the assay conditions (pH = 7.7), Q224E showed a 10\(^{-4}\)-fold decrease in its \(k_{\text{cat}}\) value compare to that of wild-type enzyme, with minor changes in \(K_m\) values for all the substrates. Q224M, on the other hand, exhibits 5-fold increase in \(K_m^{\text{IMP}}\) and 13-fold decrease in \(k_{\text{cat}}\). Interestingly, \(k_{\text{cat}}\) versus pH profiles revealed that pKa of the mutant enzyme exhibited a 1.5 pH decrease relative to that of the wild type enzyme. The maximum activity was observed at pH 6.2 for Q224E, and at pH 7.7 for the wild-type enzyme. Our data imply that Gln224 is a key determinant in stabilizing the transition state of the. The side chain amide group may directly form a hydrogen bond to the phosphoryl group of 6 phosphoryl IMP, besides recognizing IMP.
This is further supported by the fact that Q224E mutant enzyme is a better AMPSase at an acidic pH than at basic pH.

Another interesting feature of AMPSase is that GTP is the specific energy supplier to drive the reaction. Wild-type AMPSase has very low affinity for other nucleotide triphosphates. It is an challenging task to modify the exquisite GTP binding specificity, as that it might utilize ATP and other nucleotides. This study may provide alternative regulation mechanisms not only for AMPSase function, but also for GTP-binding proteins in signal transduction pathways. Previous studies indicated that the P-loop recognizes phosphates of GTP, and Asp333 plays a crucial role in binding the GTP base moiety (7-10). The crystal structure indicated another residue, Ser414, similar to Ser173 of EF-Tu, may form a hydrogen bond with 6-oxo group of GTP. Ser414 was replaced by Glu to introduce a carboxylate group near C6 of the purine ring in order to form a hydrogen bond with 6-amino group of ATP. \( K_m^{\text{ATP}} \) of S414E has been successfully decreased more than 10-fold, from 2mM to 150\( \mu \)M, relative to that of the wild-type enzyme, on the other hand, alanine substitution of Ser414 abolished the binding ability of ATP, indicating the essential role played by this residue in ATP recognition. S414D is being constructed to keep the size of the side chain comparable to serine, in the hope that it will increase the affinity for ATP and the catalytic efficiency. The kinetic studies were made possible by subcloning \( \text{purA} \) gene into a prokaryotic expression vector pTrc99A, which allowed the overexpression of the wild-type and mutant enzymes to constitute up to 50% of the total soluble proteins. By combining the mutations at residue 333 and 414, we are continuing to test the feasibility to utilize CTP instead of GTP as the driving force to carry out the reaction.
Dissertation Organization

This dissertation is presented as three journal papers followed by a general conclusion chapter. The references cited in the general introduction and general conclusion are listed after the general conclusion chapter.

In Chapter I of this dissertation, three conserved arginine residues were suggested to be in the vicinity of phosphates of GTP from the X-ray crystal structure. Site directed mutagenesis was utilized to investigate their roles. Kinetic analysis and computer modeling suggested that Arg303 and Arg305 may possibly form salt linkages to the α- and β-carboxylates of aspartate. Replacing Arg305 with leucine resulted in a 100-fold decrease in $k_{cat}$, implicated the importance of the positively charged side chains in the catalysis.

Chapter II will focus on the relationship among interface residues, enzyme activity, and enzyme association states. Evidences from the earlier literature raised concerns about the association states and the functional species of AMPSase. The crystal structure implies that Arg143 hydrogen bonds to the 5'-phosphoryl group of IMP in the active site of the symmetry-related subunit, and Asp231 forms intersubunit salt bridges with Lys140 and Arg147 (11, 12). R143K, R143L, D231A mutants, and the wild-type enzyme were analyzed using MALDI mass spectroscopy, sedimentation equilibrium centrifugation, and initial rate kinetics. For the first time, ligand-induced dimerization is implicated as a regulatory mechanism for AMPSase from all sources due to sequence conservation. At the same ligand concentration, the mutants with weakened subunit-subunit interaction only showed slight shifts from monomer towards dimer formation, while wild-type enzyme is
nearly 100% dimer. The possible biological significance of this finding is discussed in the text.

In Chapter III, Gln224 was identified as a critical residue in stabilizing IMP and the intermediate 6-phosphoryl IMP. The Q224E mutant enzyme is almost inactive at pH7.7, whereas the wild-type enzyme exhibits its maximum activity at this pH. Spectroscopic analysis indicated no differences in secondary structure or protein thermoinstabilities for wild-type and mutant enzymes. pH dependent kinetics indicated that the protonated β-carboxylate of Q224E increases the catalytic efficiency, suggesting the importance of the charge state at this residue. A model is built based on the kinetic data and the unligated crystal structure. Asp13 acts as a base to withdraw the hydrogen from the N-1 position under basic conditions. Gln224 may form hydrogen bonds with the 6-oxo and N-7 of IMP, thus facilitating the nucleophilicity of the 6-oxo group by stabilizing the enol ion.
CHAPTER I. IDENTIFICATION OF ARGININE RESIDUES IN THE PUTATIVE L-ASPARTATE BINDING SITE OF ESCHERICHIA COLI ADENYLOSUCCINATE SYNTHETASE*

A paper published in the *Journal of Biological Chemistry*

Wenyan Wang², Bradley W. Poland², Richard B. Honzatko², and Herbert J. Fromm²³

ABSTRACT

Three arginine residues in the putative aspartate binding site of *Escherichia coli* adenylosuccinate synthetase were changed to leucines by site directed mutagenesis. The mutant enzymes R303L, R304L, and R305L were purified to homogeneity on the basis of sodium dodecyl sulfate polyacrylamide gel electrophoresis and characterized by circular dichroism spectrometry (CD) and initial rate kinetics. CD spectra indicated no differences in secondary structure between the mutants and the wild type enzyme in the absence of the substrates. The $K_m$ values for GTP and IMP for the mutants and the wild-type

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²Two graduate students and two professors, respectively, Department of Biochemistry and Biophysics, Iowa State University. Research conducted and manuscript written by Wang with the assistance from the others.

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enzyme were values of $K_m$ for the substrate aspartate relative to the wild-type enzyme. Although comparable. However, the mutant enzymes exhibited 50- to 200-fold increases in their values of $K_m$ for the substrate aspartate relative to the wild-type enzyme. Although the $k_{cat}$ values for the mutant enzymes decreased, the changes were not as dramatic as those observed for the $K_m$ of aspartate. The modeling of aspartate in the crystal structure of the complex of adenylosuccinate synthetase with IMP and GDP$\text{Mg}^+$ is consistent with the results of mutagenesis, placing the $\alpha$- and $\beta$- carboxylates of aspartate near the side chains of Arg-131, -303, and 305.

INTRODUCTION

Adenylosuccinate synthetase (AMPSase) from *Escherichia coli* (IMP: L-aspartate ligase (GTP forming), EC 6.3.4.4.) catalyzes the first committed step in the *de novo* synthesis of AMP from IMP:

$$\text{IMP} + \text{GTP} + \text{aspartate} \rightleftharpoons \text{adenylosuccinate} + \text{GDP} + \text{Pi}$$

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).
AMPSase was cloned in 1987 (3) and the crystal structure was recently determined to a resolution of 2.8 Å(4). The enzyme is a homodimer encoded by a single gene, purA, with a polypeptide molecular weight of 48,000. Three different enzyme mechanisms have been proposed for AMPSase (1). The most widely accepted mechanism, proposed by Liberman (5) and Fromm (6), involves a 6-phosphoryl-IMP intermediate by the nucleophilic attack of the 6-oxo group of IMP on the γ-phosphorous of GTP. Adenylosuccinate is then formed by a second nucleophilic attack by the amino group of aspartate on the C-6 of 6-phosphoryl-IMP, displacing the phosphate. Hampton and Chu (7) suggested that the 6-phosphoryl group of IMP could be important for both IMP binding and catalysis. Enzyme product inhibition studies suggested that the IMP and aspartate binding sites are spatially separated but in close proximity(1,8).

X-ray crystallography studies (4) of wild-type AMPSase showed that much of the enzyme structure involved in GTP binding is closely related to other GTP binding proteins. A number of studies involving modification of putative GTP binding residues by site directed mutagenesis based upon the crystal structure of the enzyme provided evidence fully consistent with the three dimensional structure of the enzyme(9,10). However, little is known about the other two substrate binding sites. X-ray diffraction studies of the unligated enzyme(3) indicate residues 298-304 are poorly ordered. Preliminary X-ray diffraction studies of the enzyme ligated with MgGDP\(^{\dagger}\) and IMP, however, show that Arg303 approaches, but does not interact with bound IMP, that Arg304 is directed away from the IMP molecule, and that Arg305 is approximately 5 Å away from both the β-phosphate of MgGDP\(^{\dagger}\) and 6-oxo group of IMP. In order to
understand the roles of Arg303, Arg304 and Arg305, these residues were changed to Leu to remove the guanidinium groups. The mutant enzymes were purified and characterized using SDS-PAGE, CD spectra and initial rate kinetics study. All of the three mutants exhibited about the same Km values for GTP and IMP as did wild-type AMPSase; however, large increases in Km for aspartate relative to the wild-type enzyme were noted. The $k_{cat}$ values of the mutant enzymes were also much lower than wild type AMPSase. Our results strongly suggest that the three arginine residues are not part of the GTP or IMP binding sites, but rather are involved in aspartate binding and to some extent in catalysis as well. This is the first study that implicates specific residues in the binding of aspartate to AMPSase.

EXPERIMENTAL PROCEDURES

Materials—GTP, IMP, L-aspartate, PMSF, 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. B. Bachman (Genetic Center, Yale University). Unless specified otherwise, other reagents and chemicals used in the experiments were obtained from Sigma.

Site-directed mutagenesis—Recombinant DNA manipulation was performed using standard procedures (10). The plasmid containing a 1.6 kb HindIII fragment from PMS204 ligated into PUC118 was used in the mutagenesis step. All of the mutagenic
oligonucleotide primers used in the experiments were synthesized on a Bioreserach 8570EX automated DNA synthesizer at the DNA facility at Iowa State University. Mutagenesis was carried out according to the protocol provided by Amersham. The mutations were confirmed by DNA sequencing using chain termination method (11). The 1.6 kb HindIII fragment with the proper mutation was ligated back into PMSN, a plasmid from self ligation of PMS204 after its 1.6 kb HindIII fragment was removed. The newly formed PMS204 plasmids with the correct mutations were selected and then transformed into XL-1 blue cells. The plasmids isolated from that strain were used to transform E. coli strain purA~ H1238 which was then used for cell culture and protein purification.

Protein assay—Protein concentration was determined by the Bradford method (13), using bovine serum albumin as the standard. The concentration referred to monomers.

Purification of wild type and mutant adenylosuccinate synthetase—The wild type and the mutant enzymes were purified using a Phenyl Sepharose CL-4B column, a Cibacron blue 3GA column, and a DEAE-TSK HPLC column. The experimental details are described elsewhere (8,12). The purity of the enzyme was checked by SDS-PAGE according to Laemmli (13). AMPSase activity was determined as described earlier (15).

Kinetic study of the wild type and mutant AMPSase—The concentrations of the stock solutions for OTP and IMP were determined using their extinction coefficients at 253nm and 248nm, respectively. For each reaction, the increase at 288nm instead of 280nm was recorded at 23°C. For mutant enzymes, the $K_m$ for aspartate was obtained by holding GTP and IMP at the saturating concentration ($10K_m$), while the $K_m$ values for GTP and IMP were obtained by using the aspartate concentration at about 30mM. 1-80μg
of the purified enzymes were used in each kinetic assay reaction, depending on the
specific activity of the enzyme.

Circular dichroism spectrometry—Circular dichroism spectra of the wild-type
enzyme and the mutant enzymes were acquired on a JASCO J710 spectropolarimeter
equipped with a data processor. Samples (100μg/ml) were placed in 1 mm cuvettes and
data points were collected in 0.1 nm increments. Each spectrum was calibrated to remove
the background of the buffer and smoothed using the program in the computer of the
spectrometer. The data were analyzed by the JASCO analysis program or by the
computer program PSIPILOT.

RESULTS

Comparison of the sequence 301-310 in E.coli AMPSase with the synthetases from
other sources—Arg303 is conserved in all eight enzymes; Arg305 is conserved in all
except the synthetase in Bacillus subtilis; and Arg304 is not conserved (Table I).

In the X-ray crystal structure of the unligated enzyme (4), Arg303-305 are located in a
poorly organized loop, which defines one edge of the active site. Preliminary X-ray
results of the synthetase ligated with MgGDP⁻¹ and IMP clearly reveal the locations of
Arg303-305, the side chains of Arg303 and Arg305 project into the active site, but do not
directly bind to either IMP or MgGDP⁻¹. Site-specific mutagenesis studies were
undertaken in order to better understand the roles of the three arginine residues in
question.
Mutagenesis of *E. coli* AMPSase purA cDNA and purification of the mutant enzymes—The oligonucleotide primers used in mutagenesis are shown in Table II together with the sequencing primer for confirming the mutants. In our study, Arg303, 304, 305 were changed to leucines to remove the side chain guanidinium group as a means of disrupting salt bridges or hydrogen bonds while at the same time keeping the size of the side chain comparable to that of the original residue.

All the mutant enzymes were purified using procedures similar to those for wild-type AMPSase. The purity of the enzymes were evaluated by SDS-polyacrylamide gel electrophoresis. All the enzymes exhibited greater than 95% purity using electrophoresis as the criterion of purity (data not shown). The molecular mass of the single polypeptide band for each enzyme form was 48,000.

*E. coli* purA^- HI238 harboring the mutant PMS204 plasmid grew more slowly than the bacteria containing the wild-type plasmid. Given the same culture conditions, the cell yield of bacteria expressing R303L and R304L was 80% that of the *E. coli* expressing the wild-type enzyme, while R305L was only 40%. The mutations at the three arginine sites had a negative affect on the normal growth of the cells, suggesting that the mutated enzyme might exhibit some differences in properties compared to wild-type AMPSase.

Secondary structure analysis—Circular dichroism spectrometry (CD) was used to analyze the secondary structures of the mutant AMPSase and the wild type enzyme. The CD spectra of the four enzymes were superimposable (data not shown) from 200nm - 260nm. These observations indicated that the mutant residues did not disrupt global secondary structures detected by CD.
Kinetic analysis of AMPSase mutants—The kinetic parameters for GTP, IMP, and aspartate are summarized in Table III. The $K_m$ values for GTP and IMP were only marginally affected by the mutations compared to the wild-type enzyme, suggesting that Arg303, Arg304 and Arg305 are not involved in binding of the substrates GTP or IMP. However, the $K_m$ values for aspartate showed very significant increases for all three mutants compared to wild-type AMPase. The $K_m$ values exhibited by R303L, R304L and R305L were 250, 290 and 44-times greater than that of wild-type AMPSase. The changes suggest that all of the residues are involved in aspartate binding. Another kinetic parameter, $k_{cat}$, is also significantly different in the mutant enzymes relative to wild-type AMPSase. The $k_{cat}$ for R303L, R304L were 0.133/s and 0.183/s respectively, equivalent to 13.3% and 18.3% of the wild-type activity. On the other hand, in the case of R305L, a $k_{cat}$ value of 0.0082/s, is only 0.8% that of the wild-type enzyme. The changes in enzymatic activity suggest that Arg303, Arg304, and Arg305 may play a role in AMPSase catalysis as well as binding of aspartate. Comparison of the specificity constants, $k_{cat}/K_m$, is also available in Table III. For aspartate, the specificity constants decreased $5.3 \times 10^{-4}$, $6.4 \times 10^{-4}$ and $1.9 \times 10^{-4}$ for R303L, R304L and R305L, respectively, relative to wild-type AMPSase. These findings demonstrate that by changing the arginine residues 303-305 to leucine, the affinity of the enzyme for aspartate is drastically reduced. This may result from the disruption of the salt linkages or hydrogen bonds between these arginines and the
substrate aspartate and/or between these arginines and other residues important for maintaining the proper conformation for aspartate binding and catalysis.

DISCUSSION

Comparison of the cloned AMPSase cDNA sequences from eight different sources showed that Arg303 is highly conserved in all AMPSases, while Arg305 is well conserved in seven of the eight known sequences. Arg304 shows no homology. These data suggest that there must be some structural similarity among the AMPSases in this region of the polypeptide chain.

The conserved Arg-303, Arg-305 residues clearly play important roles in the enzyme catalyzed reaction. The substitution of leucine at position 303 led to a 250-fold increase of $K_m^{\text{asp}}$ compared to the wild-type enzyme, as well as a drastic decrease of $k_{cat}/K_m^{\text{asp}}$ by three orders of magnitude. On the other hand, the $K_m$ values for GTP and IMP were not greatly affected by this mutation. The R305L mutant retained only 0.8% of the catalytic activity of the wild-type. It exhibited $K_m$ values for GTP and IMP that are similar to the wild-type enzyme. Although the $K_m$ for aspartate increased 45-fold, $k_{cat}/K_m^{\text{asp}}$ decreased to 1/5000 of that of the wild-type enzyme. This significant loss of catalytic activity is consistent with our observation that the purA+ H1238 strain of *E. coli* harboring the R305L-PMS204 plasmid grew much more slowly and yielded only 40% of the cell weight compared to the bacteria harboring the wild-type plasmid. This suggests
that even though the vector allows 40-fold overexpression of AMPSase (3), replacement of Arg305 with leucine impaired the catalytic activity of the enzyme which in turn may have led to a decrease in AMP synthesis.

Isotope exchange experiments from our laboratory (24) suggested that aspartate prefers binding to the ternary enzyme complex (GTP-IMP-enzyme) rather than to the free enzyme. It is reasonable to suggest that for wild-type AMPSase, a conformational change is required to facilitate the binding of aspartate. Arg-305 is within 5Å of the carbon 6 of IMP and has the same conformation both in the presence and absence of the active site ligands, MgGDP^1 and IMP. Arg is available for direct interaction with aspartate, consistent with the decrease in $k_{cat}$ and increase in $K_m$ for aspartate, exhibited by the Leu-305 mutant. Very probably the proper orientation of Arg-305 is required for the precise alignment in the quaternary complex of enzyme, MgGTP^2, IMP, and aspartate, the breakdown of which is rate limiting in the AMPSase reaction (15). Arg-304 stabilizes the conformation of the loop consisting of residue 297 - 305 by forming a salt link to Glu281, but only in the presence of active site ligands. Hence the effect of the Leu-304 mutant on the $K_m$ of aspartate is probably indirect and due to the conformational destabilization of the 297 -305 loop. Arg303 hydrogen bonds to a loop consisting of residues 125-129. The loop folds over the ribose moiety of IMP but is disordered in the absence of the ligand. Again Arg-303 could stabilize the conformation of 297 -305 loop and thereby enhance the affinity of the enzyme for aspartate. However, as Arg-303 approaches the active site, a direct role in the binding of aspartate cannot be excluded.
One other residue, Arg131, is in position and available for a direct interaction with aspartate.

Knowing the position of bound IMP and that the amino group of aspartate must approach C-6 of IMP along a direction perpendicular to the plane of the base permits the generation of a model for the binding of aspartate (Fig. 1). The model places the α-carboxylate of aspartate in a salt link with Arg-305 and the β-carboxylate in a salt link with Arg131. The amino group of aspartate would be fixed in the vicinity of C-6 of IMP by a hydrogen bond to the carbonyl of residue 38. The model id speculative, inasmuch as we do not know the structure of the enzyme in its complex with the putative 6-phosphoryl IMP intermediate. The presence of the 6-phosphoryl IMP may trigger additional conformational changes in the enzyme, particularly in the vicinity of residues 38-40.

REFERENCES


Table I

Alignment of *E. coli* AMPSase amino acid sequence 301-310 with the sequences of the AMPSase from other sources

<table>
<thead>
<tr>
<th>Sources</th>
<th>Sequences</th>
<th>Reference</th>
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<tr>
<td><em>Bacillus subtilis</em></td>
<td>TGRPPRVGWFD</td>
<td>16</td>
</tr>
<tr>
<td><em>Dictyostelium dicoidium</em></td>
<td>TGRPRRIGWLD</td>
<td>17</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>TGRRRRTGWLD</td>
<td>18</td>
</tr>
<tr>
<td>Human liver</td>
<td>TGRKRRCGWLD</td>
<td>19</td>
</tr>
<tr>
<td>Mouse muscle</td>
<td>TGRKRRCGWLD</td>
<td>20</td>
</tr>
<tr>
<td>Mouse T-lymphoma cells</td>
<td>TGRKRRCGWLD</td>
<td>21</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>TGRKRRCGWLD</td>
<td>22</td>
</tr>
<tr>
<td><em>Thiobacillus ferrooxidans</em></td>
<td>TGRARRCGWLD</td>
<td>23</td>
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## Table II

Oligonucleotides used in site-directed mutagenesis

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Sequences of primers*</th>
</tr>
</thead>
<tbody>
<tr>
<td>R303L</td>
<td>TACGACGAA_GCCCCCGTAGTT</td>
</tr>
<tr>
<td>R304L</td>
<td>CCGGTACG AC_GACGCCCCGT</td>
</tr>
<tr>
<td>R305L</td>
<td>CTCCGGTACGA_GACGACGCC</td>
</tr>
<tr>
<td>sequencing primer</td>
<td>CTCGTGTAGGTGCAGGT</td>
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*underlined letters indicate the mismatches
Table III

Kinetic parameters of wild-type and mutant AMPSases from *E.coli*

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m^{GTP}$ (μM)</th>
<th>$K_m^{IMP}$ (μM)</th>
<th>$K_m^{ASP}$ (mM)</th>
<th>$k_{cat}/K_m^{GTP}$ (μM)$^{-1}$ $10^3$</th>
<th>$k_{cat}/K_m^{IMP}$ (μM)$^{-1}$ $10^3$</th>
<th>$k_{cat}/K_m^{ASP}$ (mM)$^{-1}$ $10^3$</th>
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<tr>
<td>Wild-type</td>
<td>1.00±0.05</td>
<td>26.2±2.30</td>
<td>27.8±1.30</td>
<td>0.23±0.04</td>
<td>38.0±3.81</td>
<td>35.8±2.52</td>
<td>4350±772</td>
</tr>
<tr>
<td>R303L</td>
<td>0.13±0.01</td>
<td>20.1±1.40</td>
<td>35.2±3.70</td>
<td>57.4±6.50</td>
<td>6.62±0.68</td>
<td>3.78±0.49</td>
<td>2.32±0.32</td>
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<tr>
<td>R304L</td>
<td>0.18±0.02</td>
<td>26.5±1.60</td>
<td>29.6±1.90</td>
<td>65.7±9.70</td>
<td>6.90±0.86</td>
<td>6.18±0.78</td>
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<tr>
<td>R305L</td>
<td>0.008±0.000</td>
<td>30.6±2.30</td>
<td>30.5±2.20</td>
<td>10.0±1.10</td>
<td>0.27±0.02</td>
<td>0.27±0.03</td>
<td>0.82±0.06</td>
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*The enzyme assay solution contained 40mM Hepes (pH 7.7), 5mM MgCl$_2$. When GTP was the variable substrate, IMP concentration was fixed at 0.45mM. Asp was fixed at 5mM for wild-type AMPSase and at 30mM for the mutants. When IMP was the variable substrate, the GTP concentration was fixed at 0.25mM, and the Asp concentration was fixed at 5mM for the wild-type AMPSase and 30mM for the mutants. When Asp as the variable substrate, GTP and IMP concentrations were fixed at 0.25mM and 0.45mM, respectively.*
Fig. 1  Stereo view of aspartate modeled in the active site of AMPSase. The starting point of the model is the preliminary P2 crystal structure of the complex of the synthetase with IMP, GTP and Mg$^{2+}$. Aspartate has been in the active site without energy minimization. All ligands are drawn with boldface lines.
CHAPTER II.  A STUDY OF *Escherichia coli* ADENYLOSUCCINATE SYNTHETASE ASSOCIATION STATES AND THE INTERFACE RESIDUES OF THE HOMODIMER*

A paper revised for publication in *Journal of Biological Chemistry*

Wenyang Wang¹, Andrea Gorrell¹, Richard B. Honzatko¹, and Herbert J. Fromm¹,²

ABSTRACT

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 twofold related subunit. Residue Arg143 was changed to Lys and Leu, and residue Asp231 was changed to Ala. The wild-type and the mutant enzymes were purified to homogeneity.

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*This research was supported in part by Research Grant NS10546 from the National Institutes of Health, United States Public Health Services, and Grants MCB-9218763 and MCB-9316244 from the National Science Foundation. This is Journal Paper no. J-16827 of the Iowa Agriculture and Home Economics Experiment Station, Ames (Project 3191).

¹Two graduate students and two professors, respectively, Department of Biochemistry and Biophysics, Iowa State University. Research conducted and manuscript written by Wang with the assistance from the others.

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and characterized by circular dichroism (CD) spectroscopy and initial rate kinetics. CD spectra indicated no difference in secondary structure among the mutants and the wild-type enzyme in the absence of substrates. MALDI 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 with an apparent molecular mass of 90 kDa while 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 $k_{\text{cat}}$ and $K_m$ values for aspartate. However, 10- to 20- and 60- to 100- fold increases in the $K_m$ values of GTP and IMP, respectively, are observed for the mutant enzymes compared with the wild-type synthetase. Changes in dissociation constants for IMP are comparable to changes in $K_m$ 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. Given the sequence conservation amongst adenylosuccinate synthetases from a wide range of sources, the observed ligand-induced dimerization of the *Escherichia coli* enzyme may be a property of all known adenylosuccinate synthetases.

Abbreviations used are AMPSase: adenylosuccinate synthetase, KP: potassium phosphate
INTRODUCTION

Adenylosuccinate synthetase (AMPSase)\(^1\) [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 \textit{de novo} synthetic pathway for purine nucleotides:

\[
\text{IMP} + \text{L-aspartate} + \text{GTP} \overset{\text{Mg}^{2+}}{\longrightarrow} \text{adenylosuccinate} + \text{GDP} + P_i
\]

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 HIV 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 \(g\)-phosphorus atom of GTP (5,6). A second nucleophilic attack by the amino group of aspartate on C6 of 6-phosphoryl IMP displaces the phosphate and forms adenylosuccinate.

AMPSase was first purified to homogeneity in 1976 from \textit{Escherichia coli} (7) and has since been purified and characterized from many sources (1). The \textit{purA} gene, which in \textit{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 2.5Å 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 Arg147 and Lys140 of the twofold related subunit. Indeed, when Lys140 and Arg147 were replaced by isoleucine and leucine, respectively, $K_m^{\text{GTP}}$ and $K_m^{\text{IMP}}$ 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.

**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. 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-kb 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 Bioresearch 8570EX automated DNA synthesizer at the DNA Facility at Iowa State University. Mutagenesis was performed according to the protocol provided by Amersham. The mutations were
confirmed by DNA sequencing using the chain termination method (17) at the Iowa State University DNA Facility. The 1.8-kb 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 AMPSase 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.

Purification 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-PAGE according to Laemmli (19). AMPSase activity was determined as described earlier (20).

Kinetic studies of wild-type and mutant AMPSase—The concentrations of stock 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 mM for wild-type AMPSase and at 12 mM for the mutants. When IMP was the variable substrate, the concentration of GTP was fixed at 300 mM, and aspartate was fixed at 5 mM. When aspartate was the variable substrate, the concentration of GTP was fixed at 300 mM, and the concentration of IMP at 450 mM for the wild-type AMPSase and 12 mM for the mutants.
To obtain the values of $K_{ia}$ and $K_{ib}$, dissociation constants for IMP and GTP, respectively, a 5X5 matrix of substrates were used with each enzyme. The enzyme assay solution contained 20 mM HEPES (pH 7.7), 5 mM MgCl$_2$, 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 et al. (21):

$$\frac{1}{v} = \frac{1}{V_m} \left[ 1 + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{ia}K_{ib}}{AB} \right]$$ (1)

It was assumed that the kinetic mechanism for the mutant enzymes was the same as the rapid-equilibrium Random mechanism described for the wild-type enzyme (5,6) and thus $K_{ia}K_{b} = K_{a}K_{ib}$.

In Eq. 1, 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 $K_{ia}$ is the dissociation constant for IMP. $K_{ib}$ is the dissociation constant for GTP.

Circular dichroism spectrometry—Circular dichroism spectra for the wild-type and mutant enzymes were acquired on a JASCO J700 spectropolarimeter equipped with a data processor. Samples, 100 mg/ml of enzyme, dialyzed against 5 mM KPi (pH 7.0), 1 mM EDTA, 1 mM b-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 analysis software or by PSIPLOT.
MALDI mass spectroscopy—All of the enzymes were dialyzed against 5 mM KPi buffer (pH 7.0), 1 mM EDTA, and 1 mM b-mercaptoethanol. Protein concentration was adjusted to 1 mg/ml. Samples of 0.5-1.0 ml were loaded with 0.5 ml 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-TOF mass analyzer in 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 AMPSase — Analytical ultracentrifugation experiments were performed using a Beckman Optima XL-A ultracentrifuge. The temperature of the rotor (AN-60 Ti) was set at 4°C. Rotor speeds were set at 10K, 14K, and 18K. Wild-type and mutant AMPSase samples were prepared in 5 mM KPi buffer (pH 7.0) at concentrations of 2.9 to 11.6 mM corresponding to absorbences of 0.2 - 0.8 (280 nm, 1 cm cuvette). Concentration dependent equilibrium sedimentation was performed with concentrations of 4.4 mM and 58 mM, corresponding to A_{280nm} readings of 0.3 and 4.0. Samples of AMPSase in the presence of ligands were prepared by dialysis overnight against 5 mM KPi (pH 7.0), 5 mM succinate, 5 mM MgCl₂, 20 mM IMP, and 20 mM 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 nm, 295 nm 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. Absorbence 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 Hold and Weischet (22) using the “IDEAL” model on the Optima XL-A Analysis Software (Version 2.0) to get the apparent molecular weight ($MW_{app}$). The partial specific volume of AMPSase, 0.737 cm$^3$/g, was calculated by the method of Cohn and Edsall (23).

Equilibrium sedimentation data for the wild-type and mutant enzymes obtained at three different rotor speeds were analyzed using 'SELF' model in multiple data set analysis program Optima XLA. The association constants ($K_a$) for the monomer-dimer equilibrium were obtained using the following equation:

$$c_r = E + c_{m,0} \exp \left[ M(1- \bar{u}r)w(r^2+r_0^2)/2RT \right] + (c_{m,0})^2 K_a \exp\left[2M(1- \bar{u}r)w(r^2-r_0^2)/2RT \right]$$  \quad (2)

Where $c_r$ is the concentration of the protein at a given radial position, $c_{m,0}$ is the concentration of monomer at a reference position, $M$ is the monomer molecular weight, $\bar{u}$ is the partial specific volume, $r$ is the solvent density, $w$ is the angular velocity, $r$ is the radial position in centimeters from the center of rotation, $r_0$ 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 non-zero baseline. The conversion of $K_a$ into the dissociation constant $K_d$ (μM) is performed as follows:

$$K_d = \left(1/K_a\right)(2/\varepsilon l K_a) = 24.2/K_a \text{ (μM)} \quad (3)$$

where $\varepsilon = 68750$ M$^{-1}$cm$^1$ is the extinction coefficient for AMPSase at 280 nm and $l = 1.2$ cm is the pathlength of the centrifuge cells.
RESULTS

X-ray crystallographic studies suggest that interface residues play an important role in maintaining the quaternary structures of AMPSase (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 AMPSase 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 sequence 138-148 and 228-235 in *E. coli* AMPSase with the synthetases from other sources—Arg143 and Asp231 are conserved in the nine known AMPSase sequences (8, 24-31) (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 subunit projects into the IMP binding site of the symmetry-related subunit of crystallographic dimers (11).

Mutagenesis of the *E. coli* AMPSase purA gene and purification 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
Mutagenesis of the *E. coli* AMPSase *purA* gene and purification 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 to 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-PAGE and a Mr 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 nm 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*<sub>m</sub> 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*<sub>cat</sub> values; however, *K*<sub>m</sub> values for GTP and IMP exhibited significant increases for the mutant enzymes relative to wild-type AMPSase. The *K*<sub>m</sub> values for R143K, R143L, and D231A showed 2-, 10-, and 20-fold increases, respectively, compared with that of the wild-type enzyme. On the
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 R143. 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 spectroscopy. 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-kDa 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), 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 (10K, 14K, 18K). 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 MW_{app}. At low concentrations (A_{330nm} = 0.1), the MW_{app} = 45 kDa, which matches the result determined by MALDI mass
spectroscopy. At higher concentrations (\(A_{280nm} \geq 0.65\)), the \(MW_{app}\) 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 A and B. At micromolar concentrations of enzyme in the absence of ligands, the \(K_d\) values are approximately 10 mM 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.

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 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, with the equilibrium greatly favoring the monomer - 70% monomer and 30% dimer at 11.4 mM wild-type enzyme, and the equilibrium shifts 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 $K_d$ 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 R143 and D231 are well
conserved in all the AMPSase sequences, the results here also suggest that AMPSase from
\textit{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, Asp231 and Arg143, which are
involved in putative subunit-subunit interactions. Arg143 from one subunit hydrogen
bonds to the 5'-phosphoryl group of IMP in the active site of the symmetry-related subunit
(33). In addition, Arg143 hydrogen bonds to a backbone carbonyl of the juxtaposed
subunit. Because Arg143 is conserved in all nine known sequences of AMPSase (8, 24-31),
we suggest that its role in the \textit{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 increase, 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 twofold symmetry is shown in Fig. 1. The guanidinium group of Arg143 bonds to the backbone carbonyl of the symmetry related subunit, as well as the 5'-phosphate of IMP (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, in which case more than one conformational arrangement between that water molecule and the lysyl side chain is possible, but only one of these arrangements is comparable to the interaction exhibited by Arg143 in the crystal structures.

Asp231, also conserved in known AMPSase sequences, forms an intersubunit salt bridge with the side chain of Lys140 and a hydrogen bond to Arg147 through its backbone carbonyl. As for the R143K and R143L mutants, D231A showed about the same $k_{cat}$ and $K_m$ for aspartate as wild-type AMPSase. However, $K_m$ values for GTP and IMP increased 20- and 60-fold, respectively, relative to the wild-type enzyme. These results are qualitatively similar to those for the R143K and R143L mutants and are consistent with the hypothesis that the dimeric form of the synthetase 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 g-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 b-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 weight of a dimer as determined by gel filtration analysis (data not shown). These observations suggest that neither Arg143 nor Asp231 are essential for dimer formation. On the other hand, position 231 and 143 mutants studied here exhibit little change in $K_d$ values for the dimer to monomer equilibrium in the presence or absence of ligands, and higher $K_m^{(IMP)}$ and $K_m^{(GTP)}$ values with little change in $k_{cat}$ values, implying that dimerization of AMPSase is required for the precise recognition and binding of substrates IMP and GTP. Assuming that the mutant enzymes retain the rapid equilibrium random ter ter mechanism of wild-type AMPSase (1), the comparable $k_{cat}$ values for the mutants and wild-type AMPSase suggest neither Arg143 nor Asp 231 play a role in the rate-limiting step of catalysis (interconversion from AMPSase-MgGTP$^{2-}$-IMP-aspartate to AMPSase-adenylosuccinate-MgGDP$^{1-}$-P$_i$).
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 monomer in the mM concentration range and substrates should shift the monomer-dimer equilibrium to 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 pM, 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\(^{2-}\) and IMP and the reaction 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 \textit{de novo} purine nucleotide biosynthesis. This scenario may represent a major control mechanism of adenylosuccinate biosynthesis.

It is also noteworthy that AMPSase from \textit{Saccharomyces cerevisiae} was identified as a single stranded DNA binding protein that specifically recognizes an autonomously replicating sequence (41). 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.
ACKNOWLEDGMENTS

We thank Siquan Luo for his help with the analytical ultracentrifuge.

REFERENCES


Table I
Alignment of *E. coli* AMPSase amino acid sequence 138-148, 228-233
with the sequences of the AMPSase from other sources

<table>
<thead>
<tr>
<th>Sources</th>
<th>Sequences&lt;sup&gt;a&lt;/sup&gt;</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>138-148</td>
<td>228-235</td>
</tr>
<tr>
<td><em>Bacillus substilis</em></td>
<td>MDKAARIGIRI</td>
<td>LDIDQGTY</td>
</tr>
<tr>
<td><em>Dictyostelium dicodium</em></td>
<td>SSKASRGGLRV</td>
<td>LDLDFGNCY</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>EDKVARRGLRV</td>
<td>LDIDHGTY</td>
</tr>
<tr>
<td>Human liver</td>
<td>SSKAARSGLRM</td>
<td>LDIDFGTY</td>
</tr>
<tr>
<td>Mouse muscle</td>
<td>SSKAARTGLRI</td>
<td>LDIDFGTY</td>
</tr>
<tr>
<td>Mouse T-lymphoma cells</td>
<td>SSKAARSGLRM</td>
<td>LDIDFGTY</td>
</tr>
<tr>
<td><em>Haemophilus Influenzae</em></td>
<td>EDKVARRGLRV</td>
<td>LDIDHGTY</td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td>EDKVGRRAIRV</td>
<td>LDNDHGTY</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>STKASRSGLRV</td>
<td>LDIDFGTY</td>
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</tbody>
</table>

<sup>a</sup>The conserved residues corresponding to K140, R143, R147, and D231 in *E. coli* AMPSase from different sources are shown in bold character.
Table II

Oligonucleotides used in site-directed mutagenesis and sequencing

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Sequences of primers&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>R143L</td>
<td>CAGACCGCGA&lt;sub&gt;AG&lt;/sub&gt;CTACTTTATC</td>
</tr>
<tr>
<td>R143K</td>
<td>CAGACCGCGTTTTGCTACTTTATC</td>
</tr>
<tr>
<td>D231A</td>
<td>TAAATCCGTGGGCGATATCCAG</td>
</tr>
</tbody>
</table>

Sequencing primer for Residue 143: CTGTGTGAAGCATGTCC
Sequencing primer for Residue 231: ATGGCTTGGCCGACATC

<sup>a</sup>Underlined letters indicate the mismatches.
Table III

Kinetic parameters of wild-type and mutant AMPSases from *E. Coli*\(^{a}\)

<table>
<thead>
<tr>
<th>Protein</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(K_{m}^{(GTP)}) ((\mu)M)</th>
<th>(K_{m}^{(IMP)}) (mM)</th>
<th>(K_{m}^{(ASP)}) (mM)</th>
<th>(K_{in}^{(GTP)}) ((\mu)M)</th>
<th>(K_{in}^{(IMP)}) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.00 ± 0.05</td>
<td>26.2 ± 2.30</td>
<td>0.03 ± 0.00</td>
<td>0.23 ± 0.04</td>
<td>38.8 ± 6.40</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>R143K</td>
<td>1.04 ± 0.01</td>
<td>58.3 ± 6.30</td>
<td>2.50 ± 0.25</td>
<td>0.26 ± 0.05</td>
<td>31.0 ± 5.92</td>
<td>1.50 ± 0.34</td>
</tr>
<tr>
<td>R143L</td>
<td>1.01 ± 0.01</td>
<td>283 ± 26.2</td>
<td>2.79 ± 0.13</td>
<td>0.34 ± 0.06</td>
<td>196 ± 37.6</td>
<td>1.26 ± 0.25</td>
</tr>
<tr>
<td>D231A</td>
<td>0.76 ± 0.02</td>
<td>503 ± 41.3</td>
<td>1.31 ± 0.23</td>
<td>0.50 ± 0.03</td>
<td>197 ± 62.3</td>
<td>20.4 ± 0.67</td>
</tr>
</tbody>
</table>

\(^{a}\)Experimental conditions as described in Experimental Procedures.
Table IV-A

Monomer-Dimer Dissociation Constants in the Absence of Ligands

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$A_{280 \text{nm}}$</th>
<th>$K_d^a (A_{280 \text{nm}})$</th>
<th>$K_d^b (\mu \text{M})$</th>
<th>Goodness of Fit$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.3</td>
<td>0.464</td>
<td>11.2</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>0.170</td>
<td>4.12</td>
<td>3.54</td>
</tr>
<tr>
<td>R143K</td>
<td>0.3</td>
<td>0.452</td>
<td>10.9</td>
<td>0.777</td>
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<tr>
<td>R143L</td>
<td>0.2</td>
<td>0.394</td>
<td>9.53</td>
<td>0.849</td>
</tr>
<tr>
<td>D231A</td>
<td>0.3</td>
<td>0.362</td>
<td>8.76</td>
<td>0.207</td>
</tr>
</tbody>
</table>

a. The dissociation constant in 280 nm absorbance units
b. The dissociation constant in mM determined by equation (3) shown in Experimental procedure
c. Goodness of fit was determined by the chi-square test (32)
Table IV-B

Monomer-Dimer Dissociation Constants in the Presence of Ligands

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$A_{280\text{nm}}$</th>
<th>$K_d^a$ (A$_{280\text{nm}}$)</th>
<th>$K_d^b$(µM)</th>
<th>Goodness of Fit$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.4</td>
<td>4.26$\times$$10^{-40}$</td>
<td>1.03$\times$$10^{-38}$</td>
<td>0.00019</td>
</tr>
<tr>
<td>R143K</td>
<td>0.4</td>
<td>0.178</td>
<td>4.31</td>
<td>1.96</td>
</tr>
<tr>
<td>R143L</td>
<td>0.4</td>
<td>0.307</td>
<td>7.43</td>
<td>2.07</td>
</tr>
<tr>
<td>D231A</td>
<td>0.4</td>
<td>0.208</td>
<td>5.03</td>
<td>0.151</td>
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</table>

a. The dissociation constant in 280 nm absorbance units
b. The dissociation constant in mM determined by equation (3) shown in Experimental procedure
c. Goodness of fit was determined by the chi-square test (32)
Fig. 1  a) Stereoview of the AMPSase dimer complexed with IMP
b) Stereoview of the interaction of IMP with Arg143
c) Stereoview of the interaction of IMP with modeled Lys143
Fig. 2 Typical MALDI mass spectrum of wild-type and mutant enzymes of AMPSase. The [MH2]+ peak at 23383, [MH]+ peak at 46634, and [2MH]+ peak at 92693.
Fig. 3a) Typical sedimentation equilibrium distribution of AMPSase in the absence of ligands.
Fig. 3 b) Apparent molecular mass of AMPSase as a function of protein concentration (in $A_{280\text{nm}}$) by the following equation:

$$M_{\text{app}} \text{(Da)} = \left( \frac{d \ln c}{dr^2} \right) \times \frac{2RT}{(1 - \bar{ur}')w'}$$

$c$ = AMPSase concentration shown as units of absorbence at 280 nm
$r$ = radial distance from center of rotor
$T$ = temperature in Kelvin
$u$ = partial specific volume
$r'$ = solution density
$w$ = angular velocity
CHAPTER III. STUDIES OF THE ACTIVE SITE AMINO ACID RESIDUES IN *Escherichia coli* ADENYLOSUCCINATE SYNTHETASE*

A paper prepared for the *Journal of Biological Chemistry*

Wenyan Wang, Zhenlin Hou, Richard B. Honzatko, and Herbert J. Fromm

ABSTRACT

Gln34, Gln224, Leu228, and Ser240 are located in the vicinity of IMP binding domain based on the crystal structure of *Escherichia coli* adenylsuccinate synthetase (AMPSase). Leu228 and Ser240 are identical in 13 known primary sequences of AMPSase, whereas Gln224 is either a glutamine or an asparagine, except in the case of *Brucella abortus* in which Gln is replaced by an arginine, and Gln34 is conserved in 10 out of 13 sequences. Mutations of these residues of *E. coli* AMPSase (Q34E, Q224E, Q224M, L228A, S240A) were carried out by site-directed mutagenesis. The wild-type and the

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1Two graduate students and two professors, respectively, Department of Biochemistry and Biophysics, Iowa State University. Research conducted and manuscript written by Wang with the assistance from the others.

2Primary researcher and author
mutant enzymes were purified to homogeneity and characterized by circular dichroism (CD) spectroscopy and initial rate kinetics. CD spectra indicated no difference in secondary structure among the mutants and the wild-type enzyme in the absence of substrates. Alterations made at residue 224 preserved the $K_{m}^{\text{GTP}}$ and $K_{m}^{\text{ASP}}$ values, and small changes for the $K_{m}^{\text{IMP}}$ value relative to that of the wild-type enzyme were observed. The $k_{\text{cat}}$ value decreased only 13-fold for the Q224M mutant enzyme; however, Q224E mutant enzyme was almost inactive (a $10^4$-fold decrease in $k_{\text{cat}}$ value) compared to that of the wild-type enzyme. The pH-dependent $k_{\text{cat}}$ profile of the Q224E mutant enzyme showed an optimum pH at 6.2, 1.5 pH unit lower than that of the wild-type enzyme. Tryptophan emission fluorescence spectra of the three enzymes under denaturing conditions indicated comparable thermodynamic stability. Another interesting mutant enzyme Q34E, exhibits a 60-fold decrease in $k_{\text{cat}}$, compared to that of the wild-type enzyme. These findings are further supported by computer modeling results which show that Gln224 may facilitate the formation of the 6-enol ion of IMP, which is a better nucleophile than the 6-oxo to initiate the reaction. A hydrogen bond between Gln34 and Gln224 side chains may act as an orientation force to guide the side chain of Gln224 in the active site. Alanine substitutions of the other two residues, Leu228 and Ser240, failed to show any significant kinetic variations relative to that of the wild-type enzyme, indicating that these residues may not be important in AMPSase function. Given the sequence conservation amongst adenylosuccinate synthetases from a wide range of sources, the observed function of Gln224 in E. coli enzyme may be a property of all known adenylosuccinate synthetases.
INTRODUCTION

Adenylosuccinate synthetase (IMP: L-aspartate ligase (GTP-forming), EC 6.3.4.4., AMPS synthetase) is a branch point enzyme in the purine nucleotide metabolism (Stayton et al., 1983). It catalyzes the first committed step in de novo AMP synthesis from IMP and aspartate, using GTP as the energy source. It has been reported to be a target for natural herbicides (Heim et al., 1995) and treatment for pediatric leukemia (DeAbreu et al., 1995). A better understanding of the active site in this enzyme can be useful in chemotherapeutic drug design against HIV or cancer (Ahluwalia et al., 1987).

The gene coding for AMPSase, purA, has been cloned from many sources (Mantsala et al., 1992; Wiesmuller et al., 1991; Wolfe et al., 1988; Powell et al., 1992; Guicheirt et al., 1991; Guicheirt et al., 1994; Fleischmann et al., 1995; Tatum et al., 1995; Schabes et al., 1993, Bouyoub et al., 1996)). The sequences show 40 - 90% identity using BESTFIT analysis of the GCG computer program, implying the potentially similar structures and functions for this enzyme from different organisms. The crystal structure of E. coli AMPSase was determined by X-ray diffraction study to 2.8 Å (Poland et al., 1993) and was later refined to 2.5 Å (Silva et al., 1995).

The chemical mechanism proposed for the synthetase by Lieberman (Lieberman, 1956) and Fromm (1958), which calls for the initial formation of an intermediate of 6-phosphoryl IMP (6-P-IMP), has received considerable support in the literature (Webb et al., 1984; Bass et al., 1984; Cooper et al., 1986). The mechanism involves a nucleophilic attack on the γ-phosphate of GTP by 6-oxo group of IMP to form 6-P IMP, whereupon the
amino group of L-aspartate (Asp) displaces phosphate from the C-6 of the intermediate. This mechanism was further supported by the direct observation of 6-P-IMP located in the IMP binding site when AMPSase was cocrystalized in the existence of IMP, GTP and Mg$^{2+}$ (Poland and Honzatko, unpublished findings). Initial rate kinetics experiments indicated that the reaction mechanism is rapid equilibrium random ter ter (Rudolph and Fromm, 1969). Isotope exchange studies (Rudolph and Fromm, 1971) implied that substrate addition involves a preferred pathway with aspartate adding to the enzyme after binding of the nucleotide substrates. Earlier kinetic studies on competitive and product inhibition showed that the competitive inhibitors for one of the substrates are noncompetitive when compared to the other two substrates, suggesting that the three substrate binding sites are spatially separated (Rudolph, Ph. D. Thesis, 1971). The findings that AMPS exhibited a competitive inhibition pattern for IMP and noncompetitive inhibition for GTP or aspartate, suggested that AMPS possibly binds to the IMP binding domain. These results also lead to the hypothesis that the residues located in the vicinity of the C-6 of IMP may potentially be important in catalysis, e.g., by stabilizing the reaction intermediate or the transition state complex. However, the details of the catalytic mechanism are still elusive. Former studies in this laboratory indicated Arg305 is involved in both recognition of Asp and catalysis of the reaction (Wang et al., 1995). X-ray diffraction and molecular modeling studies showed that Arg305 possibly interacts with Arg131, and β-carboxylate of Asp, to ensure the specific binding and precise alignment of Asp in the active site. It is possible that Arg305 is involved in the second displacement reaction, to replace the 6-phosphoryl group of IMP.
with Asp. However, the geometric location and spatial orientation of Arg305 makes it unlikely that it directly stabilize the intermediate 6-P-IMP.

This report identifies Gln224 as a key determinant in enzyme’s active domain. It is conserved in 12 out of 13 AMPSase sequences, either as a Gln or as an Asn. X-ray crystallographic studies showed that the side chain of Gln224 is disordered in the unligated enzyme structure, indicating that it may be associated with a conformational change upon binding ligand(s). This residue is suggested to form hydrogen bonds with 6-oxo and 7-N of IMP in the crystal structure (Poland, and Honzatko, unpublished findings). To further understand its role, Gln224 was replaced by glutamate and methionine, so as to change the charge state or hydrophobility of the side chain, while keeping the size comparable to glutamine. Initial rate kinetics was carried out on the wild-type and the mutant enzymes. Our findings suggested that Gln224 is a crucial residue not only for substrate binding, and more importantly, for catalysis as well.

Two other residues, Leu228 and Ser240 are also located in the putative IMP binding domain. Alanine substitution was utilized to dissect their functions. Gln34 was suggested to form a hydrogen bond with Gln224 by the computer modeling. This hydrogen bond may be important for the alignment of Gln224 in the active site. Gln34 is replaced by glutamate to study such an accessory role.
EXPERIMENTAL PROCEDURES

Materials—GTP, IMP, L-aspartate, adenylosuccinate, PMSF, 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. A E.coli purA- strain H1238 (thr-25, fhuA49, argF58, relA1, serA27, spot1, purA54, argI61) and a E. coli purB- strain H680 (fhuA2, lacY1, tsx-70, glvV44(AS), gal-6, λ-, purB51, trpC45, his-68, tyrA2, rpsL125(strR), malT1(λR), xylA7, mtlA2, thi-1) were obtained from Genetic Center at Yale University. 0.45μm PVDF membrane was obtained from Millipore Inc.. Other reagents and chemicals used in the experiments were obtained from Sigma if not specified.

Site-directed mutagenesis—Recombinant DNA manipulation was performed using standard procedures (Sambrook et al., 1989). The plasmid containing a 1.8 kb BamHI - HindIII fragment from PMS204 ligated into PUC118 was used in the mutagenesis step. The mutagenesis primers are: Q34E - tgaccgcctctgtagctgatcaaa, Q224M - atccagcagcgtccatccgctctcctc, Q224E - atccagcagcgtaccctecgcaccttc L228A - gatatccagcgcagcgtacc, and S240A - gtgtggaagcagttacgtacg. The underlined bases are the changes compared to the original sequence. The primer used to sequence the region 224-240 is: ATGGCCTGTTGCCGAACATC, while the one used to sequence Q34E is: GACGAAGGTAAAGGTAAG. All of the 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 carried out according to the protocol provided by Amersham company. The mutations were confirmed by DNA sequencing using chain termination method (Sanger et al., 1977) at the DNA facility. The 1.8kb BamHI - HindIII fragment with the right 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 which was the strain for protein purification.

Protein assay—Protein concentration was determined by the Bradford (1976) method using bovine serum albumin as the standard. The concentration referred to monomers. The molecular mass was determined by MALDI-TOF mass spectroscopy described elsewhere (Wang et al., 1996).

Purification of wild-type and mutant AMPSase—The wild-type and mutant enzymes were purified using ammonium sulfate precipitation, phenyl sepharose CL-4B, Cibacron blue affinity column, and DEAE-TSK HPLC column according to the experimental details described elsewhere (Bass et al., 1987), with the following modifications. Stepwise ammonium sulfate gradients were used to elute the enzyme from phenyl sepharose CL-4B column. The samples were loaded on the column, then eluted out stepwise by 0.6M, 0.4M, 0.2M (NH₄)₂SO₄ in 50 mM KPi. The wild-type and mutant enzymes were eluted in 0.2M fractions, except that 80% of Q224E mutant enzyme was detected in 0.4M fractions. The purity of the enzyme were checked by SDS-PAGE according to Laemmli (1970). AMPSase activity was determined by a coupled assay method described below.

Western blot—Because of the slower growth rate and the elevated expression level for the Q224E mutant enzyme compared to that of the wild-type enzyme, we performed
western blot on wild-type, Q224M, and Q224E AMPSases to test the authenticity of the mutant enzymes. Two sets of 0.5μg of each purified proteins, and 1μg AMPS lyase were separated using 12% SDS-polyacrylamide gel eletrophoresis, and then the proteins were transferred to a PVDF membrane. A rabbit polyclonal antibody (1:5000) and the standard detection techniques (AuroProbe BLPlus and intenSE BL system, Amersham) were used to visualize the signals.

Coupled assay method—UV difference spectra were obtained on GBC UV/VIS 918 spectrophotometer with a thermocell, using the program “λ scan” from 300 nm - 220 nm. The reaction buffer contained 20 mM Hepes (pH7.7), 300 μM GTP, 450 μM IMP, 5 mM MgCl₂, 5 mM ASP, excess AMPS lyase. 1μg/ml of wild-type AMPSase or 300μg/ml Q224E mutant enzyme was used in the scan. The spectra obtained at zero time was subtracted from the spectra collected at increasing time points (Fig. 1).

A coupled assay method monitoring the production of AMP was developed on the basis of UV difference spectra. Excess AMPS lyase was used in the reaction mixture to trap and convert all the AMPS into AMP. The increase in absorbance at 275 nm was monitored, using a Δε= 15.0 X 10⁶ cm²/mol. which was derived by comparing the k_cat values for wild-type enzyme using the assay method described elsewhere (Rudolph, 1971) and the method developed in this study.

Kinetic study of the wild-type and mutant AMPSase—The concentration of the stock solution for GTP and IMP were determined using their extinction coefficients at 253 nm and 248 nm respectively. The enzyme assay solution contained 20 mM Hepes (pH 7.7), 5 mM MgCl₂. When GTP was the variable substrate, Asp was fixed at 5 mM, IMP
concentration was fixed at 450 \( \mu M \). When IMP was the variable substrate, the GTP concentration was fixed at 300 \( \mu M \), and the Asp concentration was fixed at 5 mM. When Asp was used as the variable substrate, the GTP concentration was fixed at 300 \( \mu M \), while the IMP concentrations were fixed at 450 \( \mu M \).

Circular dichroism spectrometry—Circular dichroism spectra for the wild-type enzyme and the mutant enzymes were acquired on JASCO J700 spectropolarimeter equipped with a dataprocessor. Samples (100 \( \mu g/ml \)) were placed in 1 cm cuvette and data points were collected in a 0.1 nm increments. Each spectrum was calibrated to remove the background of the buffer and smoothed using the program in the computer of the spectrometer. The data were analyzed by JASCO analysis or PSI PLOT.

Intrinsic tryptophan emission fluorescence measurements—Fluorescence measurements, using 1-cm pathlength quartz cells, were carried out in a SLM 8000C spectrofluorometer equipped with computer-controlled data accumulation and processing ability. The proteins were excited at 290 nm and the emission spectra from 300 to 400 nm were recorded. Each sample contains 200 \( \mu g/ml \) protein in 20 mM Hepes buffer (pH 7.7). 0 - 1.5N guanidium HCl (GnHCl) were used in the denaturation experiments.

pH-dependent kinetics—Since the Q224E mutant enzyme was virtually inactive in the kinetic assay, we further studied \( k_{cat} \) versus pH profiles for the wild-type and the Q224E mutant enzyme. Two of the “good” buffer reagents, Mes and Hepes, were chosen to cover the active pH range (approximately 5.5-8.5) for wild type enzyme. The assay solution contains 20 mM Mes/Hepes buffer at different pH values, 300 \( \mu M \) GTP, 5 mM MgCl\(_2\), 5 mM ASP. IMP concentrations varied from 25 \( \mu M \) to 500 \( \mu M \) IMP, depending upon the
apparent $K_m^{\text{IMP}}$ values. 300 $\mu$g/ml Q224E mutant enzyme or 1 $\mu$g/ml of wild-type AMPSase with excess AMPS lyase were used in the assays. Longer assay time (5 - 10 minutes) was used for the mutant enzyme to compensate for its lower activity.

RESULTS

Sequence conservation of Gln34, Gln224, Leu228, and Ser240—AMPSase sequences from different sources were aligned using the GCG computer program. Gln224 is replaced by asparagine in 4 out of the 13 sequences, with one exception as an arginine in the AMPSase from *Brucella abortus*. These observations suggested the importance of side chain amide groups at this position (Table I).

In the crystal structure of the unligated enzyme, Gln224 belongs to a poorly ordered loop near the IMP binding domain (Poland *et al.*, 1993). The loop may undergo a conformational change to accommodate the active site upon binding ligand(s). Preliminary X-ray diffraction studies of the synthetase ligated with IMP, GTP and $\text{Mg}^{2+}$ indicates that Gln224 may directly recognize the 6-oxo and N-7 of IMP (unpublished findings). Q224M and Q224E was constructed to examine the functional roles of the amide side chain by either introducing an aliphatic group, or a carboxylate group, while keeping the size of the side chain comparable to that of glutamine.

The other two conserved residues, Leu228 and Ser240, were implicated to be in the IMP binding site (Silva *et al.*, 1995). The aliphatic side chain of Leu228 is parallel to the
purine ring of IMP, within a distance that allows for the formation of hydrophobic interactions. The hydroxyl group of Ser240 may form a hydrogen bond with 5' phosphoryl group of IMP. Alanine substitution was made at these positions to probe their functions.

The β- amide group of Gln34 was implied by the crystal structure to form a hydrogen bond with β-carbonyl group of Gln224. This interaction may affect the functions of Gln224 in the active site. Gln34 was replaced with a glutamate in order to study such a “network” effect.

Mutagenesis, expression and purification of AMPS synthetases—All the mutant enzymes were purified by using a procedure similar to that for wild-type AMPSase. The purity of the enzyme was evaluated by SDS-polyacrylamide gel electrophoresis. All the enzymes exhibited greater than 95% purity when electrophoresis is used as the criterion. The molecular mass of the single polypeptides determined by MALDI-TOF mass spectroscopy was 46kDa. Q224E grew much slower on either LB agar plates or in liquid media. 1.5 grams of cells per liter could be collected from the culture, one third that of the wild-type strain. However, 15 mg AMPSase per gram of cells was obtained after purification, a 15-fold increase in protein yield compared to that of wild-type enzyme. No activity was detected using the assay method described before (Rudolph and Fromm, 1969, Kang and Fromm, 1994). This unique growth and expression pattern, not observed for any other mutant AMPSases before, may indicate a serious disruption in the AMPSase function and thus the purine nucleotide metabolic pathway. Western blot, and complementation experiments using H680 and HI238 as the recipient strains, were used to verify the authenticity of Q224E mutant enzyme. The wild-type, Q224M and Q224E enzymes were
recognized by the antibody, while AMPS lyase was not (data not shown). The Q224M, and Q224E mutant plasmids complemented to purA' in H1238, but not purB' in H680.

**Secondary structure analysis**—Circular dichroism spectrometry (CD) was used to analyze the secondary structures of the mutant AMPSase and the wild-type enzyme. The CD spectra of the mutant enzymes were superimposable (data not shown) from 200nm to 260nm. These observations indicated that there were no disrupted global secondary structures detectable by CD for all the mutant enzymes.

**Kinetics characterization of wild-type and mutant AMPSases**—The kinetic parameters for wild-type and mutant AMPSase were determined in pH7.7 Hepes buffer (Table II). No significant changes in K_m values for GTP or Asp were observed for Q224E, or Q224M mutant enzymes relative to that of the wild-type enzyme. The Q224M mutant enzyme displays a 5-fold increase in K_mIMP, and a 13-fold decrease in k_cat value compared with that of the wild-type enzyme. On the other hand, the Q224E mutant enzyme, with only marginal changes in K_m for the three substrates, exhibited a four magnitude decrease in its k_cat value relative to that of wild-type AMPSase. The specificity constants for Q224M, k_cat/K_m, decreased 6-, 25-, and 4-fold for GTP, IMP, and Asp, respectively, relative to that of the wild type enzyme, compared with a 10^4-fold decrease of the Q224E mutant enzyme for all the substrates. These findings are not only in harmony with the prediction from the X-ray crystal structure, that Gln224 may form hydrogen bonds with IMP, but also suggest that the amide side chain of the Q224E mutant enzyme must play a more important role in the rate limiting step of the reaction.
The substitution of Gln34 with a glutamate resulted in a 60-fold decrease in $k_{cat}$ value, a much smaller decrease compared to that of Q224E mutant enzyme. It was implicated from the X-ray crystal structure that the β-amide group of Gln34 may form a hydrogen bond with the β-carbonyl group of Gln224. This hydrogen bond may direct Gln224 for a correct orientation in the active site. The mutation of Q34E may lead to an indirect disruption on the enzyme activity through residue 224. On the other hand, a 40-fold increase in $K_m^{\text{GTP}}$ was observed. The reason was not immediately obvious in our model building, since Gln34 is about 10Å away from the GTP binding domain. This residue is located on a β-sheet in the core region of the enzyme. It is possible that the substitution of the side chain may lead to a backbone disturbance, which in turn asserts a negative effect on GTP recognition.

Alanine substitution of the other two residues, Leu228 and Ser240, resulted in minor changes in the kinetic parameters. A 4-fold decrease of $k_{cat}$ value for L228A compared to that of the wild-type enzyme suggested that the aliphatic side chain of Leu228 may provide a small amount of binding energy in the reaction. Although Ser240 is located near the 5’-phosphoryl group of IMP, our kinetic data failed to indicate its functional significance in IMP binding. Since Ser240 is part of a disordered loop, the conformational change upon ligand binding may put other adjacent residues in direct contact with the 5’phosphoryl group.

Intrinsic tryptophan emission fluorescence measurements—The fluorescence emission spectra of the wild-type, Q34E, Q224M and Q224E were collected in the presence of different concentrations of GnHCl. The unfolding effects on the wild-type and mutant
enzymes were comparable. The emission maximum was 333 nm for the native enzymes in the absence of GnHCl, and was gradually shifted to 350 nm for the fully unfolded enzymes in 1.5N GnHCl. This observation indicated that mutations at residue 34 and 224 did not change the thermodynamic stabilities, relative to that of the wild-type enzyme (data not shown).

**pH effects on AMPSase activity**—Acting as hydrogen donors or acceptors, the amide side chains of glutamine and asparagine do not ionize, while the β-carboxylate of glutamate and aspartate are highly polar and ionizable easily. Replacing the amide group of Gln224 with a carboxylate group resulted in a four orders of magnitude decrease in $k_{cat}$, while the substrate affinity at the active site is only slightly affected as evidenced by the alterations in $K_m$ values. We decided to study the pH dependent kinetics for the wild-type and Q224E mutant enzymes, in hopes of defining the potential correlation of enzyme activity with the protonation states of the side chain at residue 224. Both titration curves are typically bell-shaped for $k_{cat}$ versus pH profiles (Fig. 2). The $K_m^{IMP}$ values showed a maximum 2-fold change for both enzymes under different pH conditions. The optimum pH for wild-type AMPSase is 7.7, and it shifted to 6.2 for the Q224E mutant enzyme, whose activity showed a 25-fold decrease when pH increases from 6.2 to 7.7. At pH 6.2, however, only a 20-fold decrease was observed for Q224E relative to that of the wild-type enzyme. These findings indicate that the protonated side chain of Glu224 can partially overcome the disadvantage of a negative charge carried on the side chain of Glu224 under basic conditions. Thus the hydrogen donor property at this position may be critical for the catalysis.
AMPSase catalyzes one of the indispensable reactions in cells leading to the synthesis of AMP from IMP. The mechanism proposed by Lieberman (1956) and Fromm (1958), in which 6-P-IMP is an intermediate, follows by a nucleophilic displacement on C-6 by Asp, enjoys wide supports in the literature, and is also in harmony with the recent observation in X-ray diffraction studies (Poland and Honzatko, unpublished findings). Kinetic studies of *E. coli* AMPSase demonstrated that the substrates bind to the enzyme active sites randomly (Rudolph and Fromm, 1969), and an isotope exchange experiment with mammalian AMPSase suggests that Asp preferred binding to the AMPSase-GTP-IMP complex rather than to the free enzyme (Cooper et al., 1986). Molecular modeling of the active sites of wild-type AMPSase placed Gln224 in a close contact with IMP. It was suggested that this residue may recognize IMP at its 6-oxo and N-7 position. Alignment of the 13 known AMPSase sequences indicated that the side chain amide group at this position is conserved with only one exception, the enzyme from *Brucella abortus*. These data suggest the importance of Gln224 in the structure and function of AMPSase.

This study focuses on elucidating the functional roles of residues Gln34, Gln224, Leu228, and Ser240 of AMPSase using site-directed mutagenesis and initial rate kinetics. Our results showed that mutations at residue 224 exhibited different kinetic behavior compared to those of the wild-type enzyme, correlating with the polarity and charge states of its side chains. Q224M mutant enzyme showed a 5-fold increase in $K_m^\text{IMP}$ and a 13-
fold decrease in $k_{cat}$ value, while the Q224E mutant enzyme, although it exhibits $K_m$ values comparable to the wild-type enzyme for all the substrates, has lost its catalytic activity.

Since the hydrogen bonding properties of an amide group is not likely to be affected by its pH environment, whereas glutamate is, investigation of the dependency of enzyme activity at different pH values may provide insight into the possible relationships between the protonation states of the side chain and the enzyme catalytic capacity. Q224E exhibits an optimum pH of 6.2 for the reaction, 1.5 unit lower than that of the wild-type enzyme. At pH 6.2, wild-type AMPSase retains 6.4% of its specific activity, compared to that at pH 7.7. Only a 20-fold decrease is observed for $k_{cat}$ of the Q224E mutant enzyme relative to that of the wild-type enzyme. These findings suggested that under acidic conditions, although neither of the enzymes is a good catalyst, the Q224E mutant enzyme performs better than under basic conditions. The unprotonated $\beta$-carboxylate group of the glutamate residue at pH 7.7 is detrimental to the enzyme function, and the negative effect can be partially reversed by the protonation of the same group.

Considering the abolished activity for Q224E under basic conditions, it is reasonable to suggest that the loss of catalytic activity of the Q224E mutant enzyme may simply due to competition of the $\beta$-carboxylate ion for the limited space, normally occupied by the negatively charged $\delta$ phosphoryl group of 6-P-IMP. It is also possible that the $\beta$-amide of Gln224 forms hydrogen bonds with 6-P-IMP, thus lowering the transition state energy. Another possible scenario is that Gln224 acts as a "pulling force" to stabilize the enolate form of the $\delta$-oxo group of IMP, which performs the nucleophilic attack on $\gamma$-phosphorous atom group of GTP to form the intermediate 6-P-IMP. Combining the computer modeling
results (Fig. 3), with the fact that the Q224M mutant exhibited a much higher $k_{cat}$ value than the Q224E mutant under basic conditions, strongly favors the second and third hypothesis.

The purine rings exist in different resonance structures depending on its environment. Tautomization at C-6 position of IMP may play an important role in carrying out the reaction. The amide side chain of Gln224 may act as a good proton donor, to facilitate the formation of the 6-enol ion of IMP (Fig. 4A). The deprotonated enolate anion is a stronger nucleophile for attacking the γ phosphate of GTP. The same hydrogen bond stabilizes intermediate 6-P-IMP as well. Under basic conditions, the deprotonated β carboxylate side chain of Q224E inhibits the formation of the 6-enolate anion of IMP (Fig. 4B), and the 6-oxo group is a more ineffective nucleophile. At pH 6.2, the protonated carboxylate side chain of Glu224 forms a hydrogen bond with the 6-hydroxyl group of IMP, making it a better nucleophile than 6-oxo group, however, it is less nucleophilic than the enolate anion (Fig. 4C). Another important residue, Asp13, is possibly involved in the reaction by withdrawing the proton from N-1 position of IMP, thus stabilize the binding of IMP, and provide a partial negative charge to the purin ring of IMP. This may explain the marginal changes in $K_m$ of Q224E and Q224M relative to that of the wild-type AMPSase. Asp13 is also involved in binding the divalent ion. Indeed, replacing Asp13 with alanine resulted in an inactive enzyme with no detectable activity (Kang et al., 1996). Methionine introduced at 224 possesses a hydrophobic side chain with no potential to be involved in hydrogen bond interaction. The neutral charge of the Q224M side chain makes all the above mentioned hydrogen bonding scenarios impossible. In this case, IMP is most
probably more equally distributed between the two species shown in Fig. 4A, and so both
IMP binding and catalysis are less favored to some extent.

The physiological importance of Gln224 was further supported by the unique features
of both cell proliferation and protein expression for the Q224E mutant enzyme. The kinetic
data implies a possible disruption of the essential purine nucleotide metabolic pathway,
which in turn may severely hinder the growth of the organism. The significantly higher
level of expression of the Q224E mutant enzyme may be a self-rescuing strategy of the
cells, to compensate for the damaged AMPSase, to ensure the operation of adenylate
synthesis and the purine nucleotide salvage cycle.

It is noteworthy that similar effects were observed for some other enzymes, in that the
substitution of one residue may result in a drastic change in enzyme properties. For
example, adenosine deaminase, catalyzing the conversion from adenosine to inosine,
showed less than 0.2% the $k_{cat}$ value relative to its wild-type enzyme, when Glu217 was
replaced with other residues. This glutamate residue is involved in stabilizing the transition
state of the reaction (Mohamedali et al., 1996). A mutant enzyme, E29Q, in fructose-1,6-
biphosphatase leads to a loss of cooperativity for AMP (Chen et al., 1994).

Also in good agreement with the computer modeling, the mutant Q34E exhibits a 60-
fold decrease in $k_{cat}$ value compared to that of the wild-type enzyme, supporting its roles in
restraining the orientation of Gln224 by hydrogen bonding. On the other hand, replacing
Leu228 and Ser240 with alanine results in no significant change in the kinetic data,
implying that they may not be essential amino acid residues for IMP binding or enzyme
catalysis.
REFERENCES

Ahluwalia, G., Cooney, D. A., Mitsuya, H., Fridland, A., Flora, K. P., Hao, Z., Dalal, M.,


266, 22582-22587


269, 4488-4496


Table I
Alignment of *E. coli* AMPSase amino acid sequence 33-35, 223-230, 240-241 with the sequences of the AMPSase from other sources

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequences&lt;sup&gt;a&lt;/sup&gt;</th>
<th>33-35</th>
<th>223-230</th>
<th>240-241</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>YQG</td>
<td>AQGVMLDI</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td>YQG</td>
<td>-RRALLDN</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Dictyostelium dicoidium</em></td>
<td>CQG</td>
<td>AQSTMLDL</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>YQG</td>
<td>AQGTLLDI</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>CQG</td>
<td>ANAALLDI</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>YQC</td>
<td>AQGTMLDI</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>CQG</td>
<td>ANAALLDI</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Pyrococcus species</em></td>
<td>GGV</td>
<td>TQGFGLSL</td>
<td>SK</td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>CAG</td>
<td>ANALMLDI</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>CQG</td>
<td>ANALMLDL</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Spiroplasma citri</em></td>
<td>WAG</td>
<td>AQGVMLDL</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Thiobacillus ferrooxidants</em></td>
<td>FQC</td>
<td>AQCTLLDV</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>YQC</td>
<td>AQGTLLDI</td>
<td>SS</td>
<td></td>
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</tbody>
</table>

<sup>a</sup>The conserved residues corresponding to Q34, Q224, L228, and S240 in *E. coli* AMPSase from different sources are shown in bold characters.
### Table II

Kinetic parameters of wild-type and mutant AMPSases from *E.coli*<sup>a</sup>

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{cat}$</th>
<th>$K_m^{\text{GTP}}$</th>
<th>$K_m^{\text{IMP}}$</th>
<th>$K_m^{\text{ASP}}$</th>
<th>$k_{cat}/K_m^{\text{GTP}}$</th>
<th>$k_{cat}/K_m^{\text{IMP}}$</th>
<th>$k_{cat}/K_m^{\text{ASP}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>(μM)</td>
<td>(μM)</td>
<td>(mM)</td>
<td>(μM)&lt;sup&gt;-1&lt;/sup&gt; 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>(μM)&lt;sup&gt;-1&lt;/sup&gt; 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>(μM)&lt;sup&gt;-1&lt;/sup&gt; 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.00 ± 0.05</td>
<td>53.5 ± 6.21</td>
<td>59.6 ± 4.63</td>
<td>0.35 ± 0.02</td>
<td>18.7 ± 2.33</td>
<td>16.8 ± 1.55</td>
<td>2.87 ± 0.22</td>
</tr>
<tr>
<td>Q34E</td>
<td>(1.68 ± 0.00) × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>(1.09 ± 0.02) × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>79.8 ± 7.71</td>
<td>0.52 ± 0.04</td>
<td>(1.05 ± 0.12) × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>0.21 ± 0.02</td>
<td>(3.23 ± 0.42) × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Q224M</td>
<td>0.07 ± 0.00</td>
<td>60.5 ± 7.32</td>
<td>316 ± 42.1</td>
<td>0.35 ± 0.04</td>
<td>3.64 ± 0.55</td>
<td>0.70 ± 0.11</td>
<td>0.63 ± 0.08</td>
</tr>
<tr>
<td>Q224I</td>
<td>(1.76 ± 0.13) × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>68.9 ± 6.10</td>
<td>50.5 ± 5.87</td>
<td>0.32 ± 0.05</td>
<td>(62.6 ± 7.52) × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>(8.53 ± 12.2) × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>(13.4 ± 2.50) × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.228A</td>
<td>0.28 ± 0.01</td>
<td>83.9 ± 9.24</td>
<td>137 ± 9.00</td>
<td>1.06 ± 0.10</td>
<td>3.34 ± 0.40</td>
<td>2.04 ± 0.15</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>S240A</td>
<td>0.69 ± 0.02</td>
<td>115 ± 12.9</td>
<td>82.5 ± 5.49</td>
<td>0.38 ± 0.02</td>
<td>6.00 ± 0.66</td>
<td>8.36 ± 0.57</td>
<td>1.82 ± 0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Experimental conditions as described in Experimental Procedures
Fig. 1 UV difference scans for reactions catalyzed by AMPSase using
a) the assay method described before (Rudolph, 1971)
b) the coupled assay method developed in this study
Fig. 2 $k_{\text{cat}}$ versus pH profiles of wild-type and Q224E mutant AMPSase. The $k_{\text{cat}}$ values for both enzymes were normalized for the clarity of comparison. The maximum $k_{\text{cat}}$ value is $1.59 \text{ s}^{-1}$ for the wild-type enzyme at pH 7.7, and $9.31 \times 10^3 \text{ s}^{-1}$ for Q224E mutant enzyme at pH 6.2.
Fig. 3  Stereoview of the IMP binding site. The hydrogen bonds between Gln224 and IMP is shown as dashed lines.
Fig. 4 Modeling of residue 224 in the IMP binding site. Hydrogen bonds were presented as dotted lines. R5P represents ribose 5'-phosphate. • represents one electron. The chemical structures of side chains of Asp13, Gln224 and Glu224 are illustrated.

A) Modeling of Gln224 in the active site of AMPSase.
Fig. 4 B) Modeling of Glu224 in the active site of AMPSase under the basic conditions
Fig. 4  C) Modeling of Glu224 in the active site of AMPSase under the acidic conditions
GENERAL CONCLUSION

Understanding the catalytic mechanism of AMPSase is of special interest because of its importance in purine nucleotide metabolism. The levels of AMPSase in most tissues are suggested to be related to malignant transformation but are independent of the growth rate of the tumors (6). It was reported as a target of herbicides (2), treatment of leukemia (3), and some natural antibiotics (22).

In this dissertation, fundamental questions concerning structural and function relationship for AMPSase were explored using biochemical and biophysical techniques. In our study, Arg303, Arg304, and Arg305 were found to be involved in catalysis and aspartate binding. Residue Gln224, located in IMP binding domain, was implicated in stabilizing one of the intermediate compounds in the AMPSase reaction. And for the first time, ligand-induced dimerization was suggested as a regulatory mechanism for AMPSase activity.

The three arginine residues 303 - 305 are located on a poorly ordered loop (300 loop) near the γ-phosphoryl group of GTP based on the crystal structure (12). The positive charges of the side chain guanidinium groups are potentially important for substrate affinity, enzyme catalysis, or both. Our kinetic experiments indicated that all three residues are involved in binding aspartate. The leucine mutants impaired the enzyme activity, demonstrated by the decreased $k_{cat}$ values, with R305L retaining only 0.8% of the activity of the wild-type enzyme. These findings are in harmony with the computer modeling results, which shows that aspartate binding may result in a conformational change in the loop where 303-305 are seated, and the loop may serve as a determinant element for enzyme activity and highly specific binding of
aspartate. Interestingly, the aspartate binding domains shows similarity to those in aspartate transcarbamylase, suggesting the conservation of the arginine residues in recognizing this widely used amino acid as a substrate.

NH$_2$OH is thought to be the only alternative substrate of aspartate. The two reactions showed similar UV difference spectra. The comparable specific activity ($k_{cat} = 0.45$ s$^{-1}$) and a much lower substrate affinity ($K_m^{NH_2OH} = -10^4$ M) than that of aspartate implied a magnitude decrease in catalytic efficiency utilizing NH$_2$OH as a substrate, possibly due to a lack of proper recognition elements. The Hill coefficient for Mg$^{2+}$ was determined to be 1 when NH$_2$OH was the substrate, instead of 2 when aspartate was the substrate. The loss of cooperativity for Mg$^{2+}$ further supported the earlier findings that the second Mg$^{2+}$ may form a complex with aspartate to make it a better nucleophile (23). Our on going project focuses on modifying substrate specificity in the aspartate binding domain, based on our knowledge of the crystal structure in the presence or absence of hadacidin. By replacing more residues at the same time, or simply remove the loop containing 300-306, we hope to modify the aspartate binding domain into a site with broader substrate specificity.

In Chapter II of this dissertation, the relationship between interface residues and the ligand-induced dimerization was discussed. The controversy on the association states of AMPSase, and the correlation with enzyme activity was magnified by the recent discovery from X-ray crystal structure (11,12), that E. coli AMPSase is a homodimer with two-fold symmetry between the subunits. This finding is at variance with the earlier report that the enzyme was a monomer using gel filtration as the criterion (11). Two interface residues, Arg143 and Asp231, were mutated into Lys143, Leu143, and Ala231. All of these mutant
enzymes exhibit only marginal changes in $k_{cat}$ and $K_{m}^{\text{ASP}}$, with more drastic changes for $K_{m}^{\text{IMP}}$ (60-100-fold increase) and $K_{m}^{\text{GTP}}$ (10-20-fold increase). The larger changes for mutations at position 143 supported the suggestion, that Arg143 recognizes the 5’ phosphoryl group of IMP. The breakage of salt bridges between Asp231-Lys140, Asp231-Arg147 may disturb the subunit-subunit interaction, and thus hinder the IMP binding affinity. These findings also implied a synergism between IMP and GTP binding.

MALDI-TOF mass spectra of the wild-type and mutant AMPSases indicated the existence of a 92 kDa species corresponding to the dimer of the enzyme. The equilibrium constants for dimerization were further calculated using analytical ultracentrifugation in the presence and absence of the active site ligands. While monomers exists as the major form for wild-type and mutant enzymes alike in the KPi buffer without ligands, the monomer-dimer equilibrium was shifted to almost all dimer for wild-type AMPSase by the ligands, demonstrated by a magnitude decrease in the dissociation constant for subunit association under these conditions. On the other hand, the active site ligands only produced marginal changes for the mutant enzymes. These findings correlated with the enzyme association states and interface residues with the enzyme activity. Additional convincing evidence came from the hysteries phenomenon when picomolar concentrations of wild-type AMPSase were used to start the reaction in the presence of saturating substrates concentration. Our data suggested that ligand-induced dimerization may serve as a major regulatory mechanism for AMPSase in vivo, and the association states may be important for other biological functions in the living organism, such as DNA replication. Based on information from the older literature and this study, we predict that inhibitors may be
synthesized chemically or screened from phage display libraries, to specifically prevent the
dimerization, thus inactivating the enzyme. This finding may provide some basis for cancer
and leukemia therapy.

In Chapter III of this dissertation, Gln224 was identified as a critical residue in the
AMPSase active site. Substitution of this residue with glutamate abolished enzyme activity
at pH7.7 which is the optimum pH for wild-type enzyme activity. On the other hand, the
effect was partially reversed at a lower pH. For example, the Q224E mutant enzyme
exhibits its maximum activity at pH6.2. Surprisingly, the drastic decrease in $k_{cat}$ for
Q224E accompanied only marginal changes in $K_m$ for these three substrates, relative to the
wild-type enzyme, indicating that this residue may be mainly involved in catalysis.
Another line of evidence came from studying the mutant Q224M, which shows a much
milder (13-fold) decrease in $k_{cat}$ with a small increase in $K_m^{IMP}$. Combining this
information with that from the crystal structure, suggests that Gln224 recognize the N-7 and
the 6-oxo group of IMP, and thus facilitate the formation of a better nucleophile: 6-enol ion.
The formation of such an ion is needed to overcome an energy barrier which may play a
part in the limiting the rate for the overall reaction catalyzed by AMPSase.

It was reported that allopurinol nucleotide is a poisonous substitute for IMP with
AMPSase for some protozoans (6), but not the enzyme from mammalian sources. Given
the structural differences between the inhibitor and IMP (C-N reversion on the positions 7
and 8), it implies that some structural dissimilarity in the IMP and AMPS binding domains
of AMPSase, and possibly AMPS lyase as well, from these different organisms. For
AMPSase, it is reasonable to postulate that the residue corresponding to Gln224 might be a
good candidate, due to its hydrogen bonding with N-7 of IMP. The information from comparison of the primary sequences and the 3D structures should provide more insights into the active sites for the two kinds of AMPSases, which may contribute to our understanding of catalytic mechanisms as well as the efforts in inhibitor design.

The essential function of this enzyme in living organisms is further provided by the correlation of enzyme activities and cell growth rates. R305L has less than 1% of the specific activity relative to that of the wild-type enzyme, while Q224E is virtually inactive. The cells harboring these two mutant plasmids grow much slower in the minimum and rich media, resulting in a decreased cell yield. For the Q224E mutant enzyme, however, the increase in AMPSase expression ratio per unit weight of cells may suggest that some compensation for the lower activity is necessary for the survival of the cells.
REFERENCES

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