Investigation of the GTP-binding consensus sequences in *Escherichia coli* adenylosuccinate synthetase and the enzyme's reaction mechanism

by

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To my wife and parents I dedicate this dissertation
with gratitude

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LIST OF NOMENCLATURE

AMPSase: adenylosuccinate synthetase

IMP: inosine monophosphate

GDP: guanosine diphosphate

GTP: guanosine triphosphate

Asp: aspartate

Pi: orthophosphate

AMP: adenosine phosphate

E. coli: *escherichia coli*

GTPγS: guanosine 5'-O-(3-thiotriphosphate)

XTP: xanthosine triphosphate

Lys: lysine

CD: circular dichroism

NMR: nuclear magnetic resonance

ITP: inosine triphosphate

GMP: guanosine monophosphate

Ala: alanine

To indicate amino acids in protein sequences or names of the mutant enzymes, one letter abbreviation was used.

GENERAL INTRODUCTION

The broad outlines of *de novo* purine nucleotide synthesis have been known since the late 1950s when Buchanan and coworkers (1) outlined the 10 reactions necessary to convert phosphoribosyl pyrophosphate to IMP. Since then research has been focused on each individual enzyme and its relationships to other members of the pathway. Although *de novo* purine nucleotide synthesis has been suggested as a target pathway for chemotherapy of anticancer drug designing (2), the research in this area has been limited to some extent due to small amounts of some enzymes in the cells. Recently, most of the enzymes involved in *de novo* purine nucleotide synthesis has been cloned (for a review, see 3), and some of them have been successfully overexpressed (4-7). Therefore, it is now possible to study and understand the detailed mechanisms of these enzymes, which may be useful for development of many efficacious chemotherapeutic agents.

AMPSase [IMP:L-aspartate ligase (GDP-forming), EC 6,3,4,4] (for a review, see 8) is ubiquitous in nature. It catalyzes the reversible reaction with GTP, IMP and Asp in the presence of Mg^{2+} to form adenylosuccinate, GDP and inorganic phosphate (Pi) as shown in the following reaction:

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

This is the first reaction committed exclusively to the formation of AMP from IMP using GTP as the unique energy source and it is believed to play an essential role in the *de novo* pathway of purine nucleotide biosynthesis. Adenylosuccinate can be further converted to AMP by adenylosuccinate lyase and then, to IMP by AMP deaminase to complete a cyclic process termed the purine nucleotide cycle (9) which may be important for generation of fumarate and ammonia in skeletal muscle tissues. It has been suggested that levels of

AMPSase in most tissues are related to malignant transformation but are independent of the growth rate of the tumor (8, references cited therein). In fact, AMPSase is a target molecule of an anticancer drug, 6-mercaptopurine ribonucleotide (10), and some of natural antibiotics such as hadacidin (N-formyl- N-hydroxyaminoacetic acid) (11) and alanosine (L(−)2-amino-3-(hydroxynitros- amino) propionic acid) (12).

Purified AMPSases from many sources have molecular masses ranging from 45,000 to 60,000 daltons; however, they are believed to exist as dimeric forms which are assumed to be the active enzymatic form in most cases. Also, based on their isoelectric points, AMPSase can be classified into two isozymes which are designated as M- and L-forms (13). For the rat liver enzymes, Type L isozyme (the acidic AMPSase) has a pI of 5.9 while the Type M isozyme (the basic AMPSase) has a pI of 8.9. It was proposed that there are differences in the functions of the isozymes, for instance, the acidic isozyme is primarily associated with biosynthesis of purine nucleotide, while Type M isozyme (the basic isozyme) is involved in the purine nucleotide cycle. Most of the bacterial AMPSases including the *E. coli* enzyme in this study belong to the L type isozyme.

For AMPSase, substrate utilization is generally quite specific. GTP can be partially replaced by dGTP (14) or GTPγS (15), and 2'-dIMP and β-D-arabinosyl-IMP were found to be alternative substrates for IMP with reduced activities (16). Hydroxylamine and cysteine sulfinate will substitute for Asp to significant degrees (17, 18). In the reaction mechanism of AMPSase, 6-phosphoryl IMP has been suggested as an intermediate by Lieberman in 1956 (17) and by Fromm in 1958 (19). In this mechanism, no partial reaction occurs until all substrates bind to the enzyme active site. Then, the 6-oxo group of IMP, probably, as an enolated form attacks the γ-phosphoryl group of GTP resulting in 6-phosphoryl IMP and GDP. Finally, the amino group of Asp performs another nucleophilic substitution on the C-6 atom esterified to phosphate of the intermediate, forming

adenylosuccinate and free phosphate. Consistent with this suggestion, kinetic studies of *E. coli* AMPSase demonstrated that the substrates bind to the enzyme active sites randomly (20), and an isotope exchange experiment with mammalian AMPSase suggested that aspartate preferred binding to the E·GTP·IMP complex rather than to the free enzyme (21). An NMR spectroscopic study using positional isotope exchange technique also suggested the participation of 6-phosphoryl IMP as an obligatory intermediate in the reaction mechanism of AMPSase (22).

There is an absolute requirement for divalent metal ions by AMPSase. Magnesium ion is known to be the best activator, but Mn^{2+} and Ca^{2+} and in some cases Co^{2+} and Ba^{2+} will substitute with decreased activity (23). Zn^{2+} and Cu^{2+} are potential inhibitors of the enzyme (24), but, interestingly, it has been reported that the Yoshida ascites enzyme is not inactivated by these metal ions (25). One metal ion was postulated to stabilize developing partial negative charges on the γ-phosphoryl group of GTP during nucleophilic substitution at the 6-oxo group of IMP. However, two reports seem to raise an interesting question on how many metal ions are necessary in the AMPSase reaction mechanism. In Lieberman's work (17) regarding the metal ion requirement, although 0.14 mM GTP was used, the apparent K_m value for total $MgCl_2$ was approximately 0.8 mM, which gives an unusually high value for the dissociation constant for Mg^{2+} of the GTP·Mg complex which is the true substrate form of GTP in the reaction. Also, addition of Ca^{2+}, Mn^{2+}, or Zn^{2+} at concentrations 1/100 of the Mg^{2+} level always caused inhibition (8) where no significant inhibition should be observed under these conditions considering the similar dissociation constants for these metal ions for the GTP·metal ion complexes. Based upon these results, these metal ions were suggested to perform a regulatory role. However, only one Mg^{2+} ion has been considered to be important in the reaction mechanism of AMPSase (26).

In recent years, cDNA sequences encoding AMPSase have been cloned from variable sources (27-34). Alignment of the known amino acid sequences reveals that high homology was found throughout the sequences, except in the regions of subunit contacts and the carboxy-terminal region. Surprisingly, a comparison of the amino acid sequences of AMPSases with the GTP-binding proteins showed that the AMPSases have several consensus sequences that are common for the GTP-binding domain among these proteins. The GTP-binding consensus sequences in AMPSase include: a glycine-rich phosphate-binding loop, GXXXXGK, near the N terminus of the proteins; and a guanine-specific binding region, (N/T/Q)KXD. It was reported by our laboratory that mutations at the putative P-loop region in AMPSase resulted in great alterations in the kinetic parameters (35). This result suggests that the conserved P-loop region is essential in the reaction mechanism of AMPSase as was suggested in the GTP-binding proteins (36). Also, X-ray diffraction studies (37) of crystalline AMPSase from *E. coli* revealed that although the folding patterns of Ha-Ras p21 protein and AMPSase were significantly different, the elements bound to the guanine and phosphate of GTP were similar in both proteins. These results raise an important question as to whether or not the GTP consensus sequences found in AMPSase may perform similar roles to those in the GTPase superfamily.

Despite their striking similarities in the X-ray crystal structures and the common consensus sequences for GTP binding in AMPSase and the GTP-binding proteins, the P-loop region of AMPSase has a major variation from the common P-loops of the GTPase superfamily. In fact, the P-loop of AMPSase lacks a (S/T) residue adjacent to it's carboxyl-side of Lys^{16}. This residue is invariant throughout the sequences of the GTP-binding proteins (36) where it is coordinated with Mg^{2+} ion which is essential in the GTPase reaction (38). At the corresponding position, AMPSase has a glycine residue whose side chain has no ability to coordinate to metal ions. Therefore, the function of the

P-loop of AMPSase is destined to be significantly different from those in other GTP-binding proteins. Another important difference in the P-loop of AMPSase compared to that of $p21^{ras}$ is that the P-loop of AMPSase has two lysines which are conserved while $p21^{ras}$ or adenylate kinase has only one essential lysine which is involved in transition-state stabilization through it's side-chain interaction with β- or γ-phosphate group of the nucleotide. Likewise, in AMPSase, it is not yet clear whether one or both, or none of lysine residues in the P-loop are involved in the reaction mechanism.

Since all of the GTP-binding proteins, including AMPSase, exhibit exquisite specificity for GTP, it may be possible to speculate that there may be common structural elements responsible for their specificities in the GTP-binding consensus sequences. The X-ray diffraction studies of $p21^{ras}$ (39) shows that the carboxylate group of Asp in the (N/T)KXD consensus sequence interacts with the C2 exocyclic amino group and a hydrogen atom of the N1 endo-nitrogen of the guanine base of GTP. In fact, a mutation of Asp to Asn at the consensus region in $p21^{ras}$ (40) reduced GTP binding affinity, and the corresponding mutant (D138N) in elongation factor Tu (41) which has a similar GTP-binding domain to that of $p21^{ras}$, changed the base specificity from GTP to XTP, although a comprehensive analysis of kinetics of the above mutants has not been performed. Since the superposition of the crystal structure of Ha-Ras p21 protein (39) onto the structure of AMPSase revealed that the structural elements in AMPSase are identical or similar to the phosphate-binding loop and GTP-binding site of the $p21^{ras}$ protein, it is conceivable that the carboxylate group of Asp^{333} in the (N/T)KXD consensus sequence may interact with the guanine base of GTP in a similar manner as shown in the X-ray crystal structure of Ha-Ras p21-GTP complex.

Dissertation Organization

In Chapter I of this dissertation, on the basis of the similarities in sequence and structure between GTP-binding proteins and AMPSase, it is important to determine whether or not the GTP-binding consensus sequences have similar functions. Lys^{331} was chosen to be investigated by site-directed mutagenesis because it is presumed to interact with the guanine base of the substrate in AMPSase. Also, the physical properties of one of the previously prepared mutants in the P-loop region, G15V (20), is characterized by CD spectrometry, NMR spectroscopy, and spectrofluorometry in order to study the role of the phosphate-binding region of AMPSase in some detail. From these investigations, it will be shown that the GTP-binding consensus sequence found in AMPSase play similar roles to those of the GTPase superfamily.

Cherter II will be devoted to investigation of the Asp residue in the $TKLD^{333}$ box, another GTP-binding consensus sequence found in AMPSase. In this section of the dissertation, the residue at the position 333 of *E. coli* AMPSase is identified as a key determinant in the recognition of nucleoside triphosphates, and by modest changes in the side chain of the residue 333, the wild-type, GTP-hydrolyzing enzyme is transformed into an even more proficient XTP-hydrolyzing enzyme. These results represent the first quantitative evaluation of the altered specificity of purine nucleotides in GTP-binding proteins. Since the mutations at this residue have effects on catalysis as well as on substrate specificity, utilization of the potential binding energy of substrates (42) is considered in the relationship between the interaction of Asp^{333} with guanine base of GTP and the catalytic activity of the AMPSase.

In the third chapter of this dissertation, a stoichiometric study of metal ions in the AMPSase reaction will be presented using kinetics to reveal, for the first time, that a

second metal ion is involved in the reaction mechanism of AMPSase. In order to understand it's role in the enzyme reaction, the relationship of the second metal ion to substrates will be established. Also, based upon this finding, the inhibition of AMPSase by other metal ions will be illustrated and the biological significance of these results will be presented.

Chapters I, II and III have been separately published in scholarly journals. GENERAL CONCLUSION following the third chapter will be devoted to a summary of the studies presented in this dissertation, and a discussion of the features of the reaction mechanism of AMPSase including some aspects of the current research. Finally, a list of the references cited in GENERAL INTRODUCTION and GENERAL SUMMARY sections will follow.

CHAPTER I. CHARACTERIZATION OF THE PUTATIVE GTP-BINDING SITE RESIDUES OF *ESCHERICHIA COLI* ADENYLOSUCCINATE SYNTHETASE BY SITE-DIRECTED MUTAGENESIS

A paper published in Archives of Biochemistry and Biophysics[1]

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ABSTRACT

Adenylosuccinate synthetase contains amino acid sequences in its GTP binding domain that are homologous to other G-proteins. This homology includes a glycine-rich phosphate-binding loop, GXXXXGK, and a guanine-specific binding region, (N/T/Q)KXD; however, virtually no other sequence homology exists between other G-proteins and adenylosuccinate synthetase. It was found from X-ray diffraction crystallography that the GTP binding domains of the $p21^{ras}$ proteins and adenylosuccinate synthetase are superimposable, and we chose therefore to study the G15V mutant, a

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phosphate-binding loop mutant, and K331L, R, two mutants of Lys^{331} which are involved in guanine ring binding. The K_m values for GTP of adenylosuccinate synthetase mutants, K331L and K331R, when compared to those of the wild-type enzyme, were 27-fold and 20-fold increased, respectively, without any significant change in the K_m values for IMP. Because both mutations affected the K_m values for GTP similarly, whereas the k_{cat} and secondary structure were essentially unchanged, it is suggested that Lys^{331} is located in the GTP-binding site of adenylosuccinate synthetase and the terminal ε-amino group of the Lys is not necessarily important in GTP-binding on the enzyme. Therefore, Lys^{331} may interact with GTP through hydrophobic interactions between its linear side chain and the aromatic ring of the guanine base of GTP. Also, structural characterization of the G15V mutant was carried out using circular dichroism (CD) spectrometry, NMR spectroscopy and spectrofluorometry. The CD spectral data indicated that the secondary structure of the G15V mutant was significantly altered by GTP and IMP, whereas that of the wild-type enzyme is not changed; however, the two enzymes exhibited similar secondary structures in the absence of substrates. The NMR spectra of both enzymes were also similar in the absence of substrates. The dissociation constant (K_d) for IMP of the G15V mutant was 8-fold larger than its K_m value which was 1.5-fold increased compared to the wild-type enzyme. From these findings, it was concluded that the phosphate-binding region of adenylosuccinate synthetase is involved in a conformational change induced by GTP and IMP binding, and that GTP and IMP binding depend on the pre-existence of other substrates at the active site of the enzyme. These results suggest that the Lys^{331} of adenylosuccinate synthetase may play similar roles in the function and structure to that of GTP-binding proteins. However, the phosphate-binding loop has additional functions besides the interaction with the γ-phosphate of GTP.

INTRODUCTION

Guanine nucleotide-binding proteins play important roles in many cellular processes (1–4) and have a common structural design and shared molecular mechanism that make up the GTPase superfamily (5,6). Striking similarities are found in the amino acid sequences among all members of this family of proteins (7). This homology includes: a glycine-rich phosphate-binding loop, GXXXXGK, near the N terminus of the proteins; a Mg^{2+} and nucleotide phosphate-binding region, (D/E)XX(G/A); and a guanine-specific binding region, (N/T/Q)KXD. Some homologous sequences are also found in enzymes that use GTP as substrates or energy sources (8,9) including adenylosuccinate synthetase (AMPSase[a]) (10). On the other hand, aside from those homologous sequences involved in GTP binding, very little sequence similarity exists between adenylosuccinate and other G-proteins.

AMPSase [IMP:L-aspartate ligase (GDP-forming), EC 6,3,4,4] (AMPSase) (for a review, see 11) catalyzes the reversible reaction with GTP, IMP and Asp in the presence of Mg^{2+} to form adenylosuccinate, GDP and inorganic phosphate (*Pi*). This is the first reaction committed to the formation of AMP from IMP using GTP as an energy source and is believed to play an important role in the *de novo* pathway of purine nucleotide biosynthesis. cDNA sequences encoding AMPSase have been cloned from *Escherichia coli* (10), mouse muscle (12), *Dictyostelium discoideum* (13) and *Bacillus subtilis* (14). Alignment of the four known amino acid sequences reveals that most of the homologous

[a] The abbreviations used are: AMPSase, adenylosuccinate synthetase: β-ME, 2-mercaptoethanol: DEAE, diethylaminoethyl: CD, circular dichroism: SDS PAGE, sodium dodesyl sulfate polyacrylamide gel electrophoresis.

[b] The synthesis of the primers in this study and the DNA sequencing of the mutated plasmids were done by the Iowa State University Nucleic Acid Facility.

sequences related to the GTP-binding site of the GTPase superfamily are conserved in all AMPSase enzymes. For example, a glycine-rich sequence GDEGKGK is found in the N-terminal region, and DXXG and TKXD are also found in the sequences of AMPSase. Studies of the $p21^{ras}$ proteins, which are involved in growth-promoting signal transduction (1), have shown that mutations in sequence conserved regions significantly affect the GTPase activity or nucleotide-binding affinity (15–18). Poland et al. (19) recently determined the structure of *E. coli* AMPSase by X-ray diffraction to 2.8 Å. The superposition of the crystal structure of Ha-Ras p21 protein onto the structure of AMPSase revealed structural elements in AMPSase that are identical or similar to the phosphate-binding loop and binding site of the $p21^{ras}$ protein. However, whether or not the consensus sequences found in AMPSases have a similar function relative to GTP-binding proteins requires further investigation.

Because the structure of adenylosuccinate synthetase ligated to substrates is not yet available, we used the known structure of $p21^{ras}$ (6) as a model for the putative AMPSase·GTP complex. On the basis of the similarities in sequence and structure between GTP-binding proteins and AMPSase, we chose to investigate by directed mutation the critical lysine, Lys^{331}, residue presumed to bind the guanine base of the substrate in AMPSase. Also, we investigated the physical properties of one of the previously prepared mutants, G15V (20), by CD spectrophotometry, NMR spectroscopy, and spectrofluorometry in order to study the role of the phosphate-binding region of AMPSase in some detail.

MATERIALS AND METHODS

Materials — *E. coli* strain XL1-Blue was purchased from Stragene, a site-directed mutagenesis kit from Amersham, and restriction enzymes were obtained from Promega.

All reagents, including the Ciba-cron Blue column material, were obtained from Sigma, and *pur* A^- strain H1238 was a gift from Dr. B. Bachman (Genetic Center, Yale University).

Site-directed Mutagenesis — Recombinant DNA manipulation was performed using standard procedures (21). A 1.6 kb Hind III fragment from PMS204 was ligated into the corresponding site in puc118, and the ligation mixture was used to transform competent TG1 cells on LB plates containing 40mg/ml ampicillin. After incubation of the plates at 37 °C overnight, six of the resultant colonies were chosen, and their plasmids were isolated to check the orientation and the size of the insert with digestion by restriction enzymes. A colony with the correctly sized and oriented plasmid was grown in the presence of M13 helper phage M13KO7, which generated single stranded DNA. The single-stranded DNA was used as a template in site-directed mutagenesis experiments. Mutagenic primers were prepared at the DNA Facility[b] at Iowa State University by using a Biosearch 8570EX automated DNA synthesizer. The primers were 5′-ACGTCCAGTA*G*AGTCAGGCA for the K331L mutant and 5′-ACGTCCAGTC*TAGTCAGGCA for the K331R where the asterisks indicate the mismatched bases. Mutagenesis was carried out according to the protocol provided by the Amersham company, which included primer extension and ligation steps. The recombinant plasmids were used to transform *E. coli* strain TG1, and more than three colonies were chosen for the sequencing which was carried out using Applied Biosystem model 373A. The sequencing primer, 5′-CGGTAAGCCACGCAGAGT, was designed to start the sequencing from 32-bases downstream of the mutated region. The nucleotide sequence analysis was done using the chain-termination method (22). The Hind III fragments encoding the mutations were ligated back into the PMS204 plasmid which was previously digested by Hind III and purified. TG1 cells were transfomed with the ligated products and the colonies containing

plasmids with the correct orientation and size (as confirmed by digestion with EcoR I or Hind III) were selected. The plasmid purified from each selected colony was used to transform *E. coli* strain XL1-Blue. Finally, *E. coli pur A*$^-$ strain H1238 was transformed with the plasmids isolated from the transformed *E. coli* XL1-Blue cells. The transformed H1238 cells were used for the purification of the corresponding mutants.

Preperation and Kinetics of Wild-Type and Mutant AMPSase — The mutant forms of AMPSase were purified using the procedure described previously (23) except that the Phenyl-Sepharose chromatography step was replaced with a Ciba-cron Blue column (1.5 cm × 20 cm). After the $(NH_4)_2SO_4$ precipitation step, the resuspended extracts were dialyzed against 5 mM KPi buffer (pH 7.0) containing 1mM EDTA and 1 mM β-mercaptoethanol (β-ME)[2], and then applied to the Ciba-cron Blue column equilibrated with the same buffer. The column was washed with 5 bed volumes of the buffer, and then with the same buffer solution containing 1 M KCl to elute AMPSase. The fractions with the enzymatic activity were concentrated with an Amicon concentrator (membrane YM 30), and dialyzed against 50 mM KPi buffer containing 1mM EDTA and 1 mM β-ME. The dialyzed proteins were applied to a DEAE HPLC column as described previously (23). Protein fractions were analysed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (24). The concentration of the purified proteins in solution was determined by the Bradford method (25), and the concentrations refer to the monomers. AMPSase activity was determined as described earlier (26). The concentrations of the stock solutions of the nucleotides were determined using their extinction coefficients at the proper wavelengths. In the case of wild-type and G15V mutant, K_m and V_{max} values for each substrate were obtained by keeping the other two substrates at saturating levels (3 to 10 times of their K_m values) and varying the third substrate concentration.

Circular Dichroism Spectroscopy — Circular dichroism spectra were acquired at room temperature on an AVIV circular dichroism spectrometer model 62DS kindly supplied by Dr. Earl Stellwagen at the University of Iowa. Samples (0.119 to 0.216 mg/ml) were placed in a 1 mm cuvette, and data points were obtained from 200 to 260 nm in 0.5 nm increments. Each spectrum was calibrated to remove the contributions of the buffer and nucleotides and smoothed using a program provided in the computer of the spectrometer.

^{1}H-NMR Spectroscopy — Samples of AMPSases were prepared for NMR by exchanging H_2O for D_2O by centrifugal ultrafiltration in Centricon-10 devices (Amicon). Three cycles of concentration and dilution with 20 mM Hepes in D_2O were used to exchange amide protons for deuterons. Final protein concentrations were 0.105 mM. Quoted pH value is direct meter reading uncorrected for the small isotope effect. ^{1}H NMR spectra were recorded at 15 °C in a Varian VXR-500 NMR spectrometer.

Spectrofluorometry — The determination of K_d values of wild-type and mutant AMPSase by spectrofluorometry was carried out using a SLM 8000C spetrofluorometer at 22 °C following the procedure previously described (27). Concentrations of protein were 0.5 to 1 μM in 25 mM Hepes at pH 7.70. Samples were excited at 290 nm and the intensity of emission at 333 nm was used for the determination of K_d values using the computer program ENZFITTER (28).

RESULTS

Mutagenesis of AMPSase cDNA and Purification of the Mutants — Table I shows the comparison of the N(T)KXD and GXXXXGK consensus sequences in the p21^{ras} proteins and the corresponding regions of AMPSase from four different sources. The

region including Lys^{331} in *E. coli* AMPSase is highly conserved in all three enzymes except the enzyme from *B. subtilis* in which it corresponds to the (N/T)KXD region of the $p21^{ras}$ proteins. Therefore, mutations at the conserved Lys is expected to affect GTP binding to AMPSase as is the case with the $p21^{ras}$ proteins (29). In this study, Lys^{331} was changed to leucine and arginine. The leucine mutant (K331L) was prepared to remove the terminal positive charge of lysine while maintaining the long alkyl side chain moiety of the lysine residue, whereas the arginine mutant (K331R) was prepared to retain the terminal positive charge. The G15V mutant had been generated previously (20).

Purification of the mutants was similar to the previously described protocol (23). In this study, the procedure was modified to improve the yield and stabilization of the proteins during purification. Because the G15V mutant bound to the Phenyl-Sepharose column tightly, it was necessary to use H_2O for the elution of the protein instead of 5mM K*Pi* buffer used for wild-type AMPSase. Unfortunately, this alteration gave rise to contamination by other proteins and inconstant results. Therefore, the Phenyl-Sepharose column was replaced with a Ciba-cron Blue column which bound wild-type AMPSase and all of the mutants at low salt concentration (5 mM K*Pi* only). The AMPSase activity was eluted at high salt concentration (5 mM K*Pi* and 1 M KCl). The second peak exhibited enzyme acitivity, whereas the first peak contained proteins that had no detectable AMPSase activity. The collected fractions from the second peak exhibited more than 50 % purity as determined by SDS–PAGE for mutant enzyme in this study as well as wild-type AMPSase. These partially purified proteins were applied to a DEAE-HPLC column (23), and highly purified proteins, as revealed by SDS–PAGE (data not shown), were obtained. The contamination of mutant enzyme by wild-type AMPSase was not possible because *E. coli* strain H1238, used to prepare all of the mutants in this study, does not express intrinsic AMPSase activity.

Kinetic Analysis of AMPSase Mutants (K331L, K331R, and G15V) and the Determination of Dissociation Constants by Fluorescence Spetroscopy — Table II summarizes the kinetic parameters and the dissociation costants (K_d) for IMP of the mutant and wild-type AMPSases. The k_{cat} values for K331L and K331R mutants were 0.25/sec and 0.32/sec, respectively, which are equivalent to about 42 % of the wild-type activity. Thus, the mutations at position 331 have only a modest effect on catalysis. However, K_m values for GTP were 587 μM for K331L and 443 μM for K331R. These values are 27- and 20-fold greater than the K_m value for GTP of wild-type AMPSase, whereas the K_m values for Asp were 20- and 5.1-fold higher for the K331L and K331R mutant, respectively. However, the K_m values for IMP were little affected by these mutations compared to the wild-type enzyme (about 2-fold increases). The k_{cat} for the G15V mutant decreased by 15-fold compared to wild-type AMPSase, whereas the K_m values for GTP, IMP and Asp increased only slightly. The K_m value of GTP for the K331R mutant was similar to that of the K331L mutant indicating that the terminal positive charge of Lys^{331} is not important in the interaction between Lys^{331} and GTP.

The dissociation constants (K_d) for IMP were 37.7 μM and 169 μM for wild-type and G15V AMPSases, respectively. Compared to the K_d value for wild-type AMPSase, the K_d value of the G15V mutant is increased (about 8-fold).

Circular Dichroism Spetroscopic Studies on the Mutant and Wild-type AMPSases in the Presence and Absence of GTP or IMP — The CD spectra of wild-type and mutant AMPSases in the absence of substrates are superimposable (data not shown). The CD spectrum of wild-type AMPSase exhibits well developed double minima at 208 nm and 222 nm which is a typical feature of the CD spectrum of α-helices (30). Wild-type AMPSase seems to have a significant α-helix content in its secondary stucture [Poland et al. (19) determined that 33 % of all residues of AMPSase are in helices]. Wild-type, K331L,

K331R and G15V AMPSases exhibit almost identical CD spectra which indicates that mutants of Lys^{331} and Gly^{15} lead to no descernable secondary structural changes despite noticeable differences in the kinetic parameters of the mutants compared to wild-type AMPSase.

CD spectra in the presence of GTP or IMP are essentially identical with that of AMPSase in the absence of these substrates, which is also the case with the K331L mutant (data not shown). However, the CD spectra of the G15V mutant in the presence of IMP or GTP are quite different compared to the G15V mutant in the absence of these nucleotides (Fig. 1). Clearly, the secondary structure of G15V is altered by either GTP or IMP binding. These altrations of G15V AMPSase in the presence of IMP or GTP are probably due to the bulky side chain of Val which replaces a β-hydrogen atom of Gly in wild-type AMPSase. Also, it should be pointed out that the regions altered by each substrate look different because the change induced by GTP could not be superimposed on the spectrum obtained with IMP. The alteration of the secondary structure of G15V mutant by IMP and GTP may be responsible for its poor activity.

^{1}H-NMR Specrum of WT and G15V AMPSases — Fig. 2 depicts the *^{1}H-NMR* aromatic regions of wild-type and G15V AMPSases. The two spectra show only minor differences implying that the mutation at the Gly^{15} (G15V) resulted in no significant distruption in the environment of those aromatic residues as observed by ^{1}H-NMR spectroscopy. The NMR results indicate that the Val mutation at Gly^{15} does affect the tertiary structure of the protein to some extent; however, these alterations are not interpreted to be global in nature.

DISCUSSION

The studies presented in this report were undertaken in an attempt to evaluate the structure-function relationships of consensus sequence residues in the phosphate-binding loop and the putative purine base-binding domain of *E. coli* AMPSase. The literature associated with the $p21^{ras}$ proteins (6), as well as the three-dimensional structure of *E. coli* AMPSase (19), suggest the importance of these residues in substrate-binding and catalysis.

A point of concern relative to wild-type and mutant enzymes is associated with the question of whether the resultant alteration in the structure of the mutant is localized or global in nature. CD spectra (data not shown) and Fig. 2 show that the global structure of G15V mutant AMPSase is similar to the wild-type enzyme in the absence of substrates. Structural alterations of the type observed with the G15V mutant in the presence of either substrate (Fig. 1) was not observed in the wild-type enzyme. On the other hand, AMPSase K331L and K331R mutants did not show any significant change in their CD spectra in the presence and absence of substrate, and in k_{cat} values compared to the wild-type enzyme, although significant changes in K_m values for GTP and Asp were observed. Based upon these results, it is possible to assume that the mutational effects in this report were not due to global changes of the enzyme structure.

The results in Table II suggest that the P-loop region and Lys^{331} of *E. coli* AMPSase may be involved in the GTP-binding site as is the case with the $p21^{ras}$ proteins (6). Recently, the three-dimensional structure of *E. coli* AMPSase was determined by X-ray diffraction techniques in the absence of substrates (19). The structure shows that the major portion of the GTP-binding region in AMPSase is superimposable on that of the $p21^{ras}$ protein including the phosphate-binding loop region, and the consensus sequence, NKXD, which is important in GTP specificity and its binding affinity. These structural

similarities between GTP-binding regions in *E. coli* AMPSase and the $p21^{ras}$ protein are consistent with our results from site-directed mutagenesis and kinetic or CD spectroscopic studies.

The crystal structure of $p21^{ras}$ (6) indicates that Lys^{331} is associated with the guanine moiety of GTP through hydrophobic, rather than ionic, interactions. In the case of the mutations at Lys^{331} of AMPSase, K_m values for GTP are increased 27-fold and 20-fold in K331L and K331R mutants, respectively, compared to the wild-type enzyme, without significant change in K_m values for IMP. It is an interesting question as to why the K331R and K331L mutants have similar K_m values for GTP although the former mutant retains a terminal positive charge like wild-type AMPSase. Also, both mutants have long-branched side chains instead of linear alkyl chains like the wild-type enzyme at position 331. Based upon these findings, it is likely that in the interaction between Lys^{331} and GTP, the terminal ε-amino group of Lys^{331} may not be important *per se*, but rather the loss of the linear alkyl chain may be responsible for the increased K_m values for GTP in both mutants. in addition, the posibility cannot be discounted that substitutions at Lys^{331} causes alteration in the ϕ and ψ angles at position 331 that are not conducive to GTP binding.

Isotope exchange experiments from our labolatory (31) suggested that Asp seemed to prefer binding to the ternary enzyme complex (GTP·IMP·E) rather than to the free enzyme. This finding implies that Asp binding may be affected by GTP or IMP preoccuping the active site of the AMPSase. This suggestion may provide a clue for the concomitant increase of GTP and Asp K_m values by mutations at Lys^{331}. However, further experiments are necessary to confirm this suggestion.

The fact that the alteration of the secondary structure of the G15V mutant can be seen only in the presence of either IMP or GTP binding (Fig. 1) suggests that the P-loop region of this mutant is involved in conformational changes that accompany substrate-

binding. In wild-type AMPSase, similar conformational changes induced by substrate-binding are believed to occur but they can not be observed in the CD spectrum. However, conformational changes of wild-type AMPSase during substrate or product binding have been found in our laboratory based on quenching of intrinsic tryptophan fluorescence by ligands (27). Also, the NMR spectrum of water-exchangable protons in AMPSase (unpublished results) shows that IMP and GTP induce structural change in wild-type AMPSase. Therefore, it is likely that the phosphate-binding loop region of wild-type AMPSase is involved in conformational changes that attend GTP and IMP binding. These findings imply that the role of the phosphate-binding loop of the AMPSase may be different compared to that of $p21^{ras}$ where the role of the P-loop is mainly limited to its interaction with the γ-phosphate of GTP. Furthermore, a comparison of the Michaelis constant (K_m) and the dissociation constant (K_d) for IMP in Table II supports the suggested role of the P-loop in the AMPSase. In the random ter-ter kinetic model suggested for AMPSase (26), the K_m value represents the dissociation constant for IMP from the quaternary complex (E·GTP·IMP·Asp), whereas the K_d value for IMP is the dissociation constant of IMP from the binary complex(E·IMP) as shown in the following equations:

$$K_m : \text{E·GTP·IMP·Asp} \rightleftharpoons \text{E·GTP·Asp} + \text{IMP}$$

$$K_d : \quad \text{E·IMP} \rightleftharpoons \text{E} + \text{IMP}$$

The K_d value for IMP of the G15V mutant is about 4.8-fold larger than the K_m value for IMP which implies that IMP binding to the G15V mutant is facilitated by other substrates bound to the enzyme. Also, in wild-type AMPSase, a similar observation was reported, but with a smaller K_d/K_m ratio (27). These results suggest that the phosphate-binding loop of AMPSase is involved in both GTP and IMP binding which are synergistic.

In conclusion, the results from site-directed mutagenesis experiments followed by spectroscopic analysis of the generated mutants suggest that the GTP binding domain of AMPSase is substantially similar to the $p21^{ras}$ proteins, presumably, resulted from convergent evolution since, aside from the GTP-binding region, very little sequence homology exists between AMPSase and other G-proteins. This suggestion is supported by comparison between the three-dimensional structures of AMPSase and the $p21^{ras}$ proteins (19). Lys^{331} may interact with GTP through hydrophobic interaction between the linear aliphatic side chain of the lysyl residue and the aromatic ring system of the guanine base of GTP as is the case with $p21^{ras}$ (6). The putative phosphate-binding loop of AMPSase may be involved in conformational changes that accompany phosphoryl-group transfer during the reaction, and in conformational changes induced by GTP and IMP binding, which make the P-loop of AMPSase distinct from those of other GTP-binding proteins such as the G-proteins.

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Table I. Comparison of Amino Acid Sequences from *E.coli* and Other Adenylosuccinate Synthetases to the GTP-Binding Consensus Sequences in the $p21^{ras}$ Protein.

Enzymes	Residues[a]	
$p21^{ras}$ protein	^{10}GAGGVGKS	^{116}NKCD
E. coli AMPSase[b]	^{12}GDEGKGKI	^{330}TKLD
Mouse muscle AMPSase[c]	^{42}GDEGKGKI	^{362}TKLD
D. discoideum AMPSase[d]	^{13}GDEGKGKI	^{329}TKLD
B. subtilis AMPSase[e]	^{12}GDEGKGKI	^{339}NSID

[a] The number shown at the left of the sequences are counted from N-terminal of each protein.

[b] Wolfe and Smith (1988).

[c] Guicherit *et al.* (1991).

[d] Wiesmüller *et al.* (1991).

[e] Mäntsälä and Zalkin (1992).

Table II Kinetic and Binding Parameters of Wild-Type and Mutant Adenylosuccinate Synthetases from *E. coli*[a]

Proteins	k_{cat} (/sec)	$K_{m,GTP}$ (μM)	$K_{m,IMP}$ (μM)	$K_{m,Asp}$ (μM)	$k_{cat}/K_{m,GTP}$ $10^{-3}(sec\mu M)^{-1}$	$k_{cat}/K_{m,GTP}$ $10^{-3}(sec\mu M)^{-1}$	$k_{cat}/K_{m,GTP}$ $10^{-3}(sec\mu M)^{-1}$	$K_{d,IMP}$ (μM)
Wild-type	0.59 ± 0.10	22 ± 5	21 ± 2	280 ± 40	27 ± 6	29 ± 6	2.1 ± 0.5	37.7 ± 2.1
K331L	0.25 ± 0.04	600 ± 100	41 ± 5	5400 ± 900	0.42 ± 0.09	6.0 ± 1.2	0.046 ± 0.020	—[b]
K331R	0.32 ± 0.08	440 ± 51	47 ± 10	1500 ± 200	0.86 ± 0.2	5.3 ± 2.2	0.17 ± 0.05	—[b]
G15V	0.039 ± 0.003	25 ± 4	35 ± 3	350 ± 50	1.6 ± 0.27	1.1 ± 0.1	0.11 ± 0.02	169 ± 17.6

[a] The enzyme assay solution contained 40 mM Hepes, pH 7.57, 10 mM $MgCl_2$. When GTP was used as the variable substrate, IMP concentration was fixed at 0.45 mM and Asp was fixed at 5 mM for wild-type and G15V mutant adenylosuccinate shnthetase and 25 mM for the K331L and K331R mutant enzymes. When IMP was the variable substrate, GTP was fixed 0.45 mM for wild-type and G15V mutant enzymes and 1.5 mM for K331L and K331R mutant enzymes. When Asp was the variable substrate, GTP was fixed 0.45 mM for wild-type and G15V mutant enzymes and 1.5 mM for K331L and K331R mutant enzymes, and IMP was fixed at 0.45 mM for all of the enzymes.

[b] Not determined.

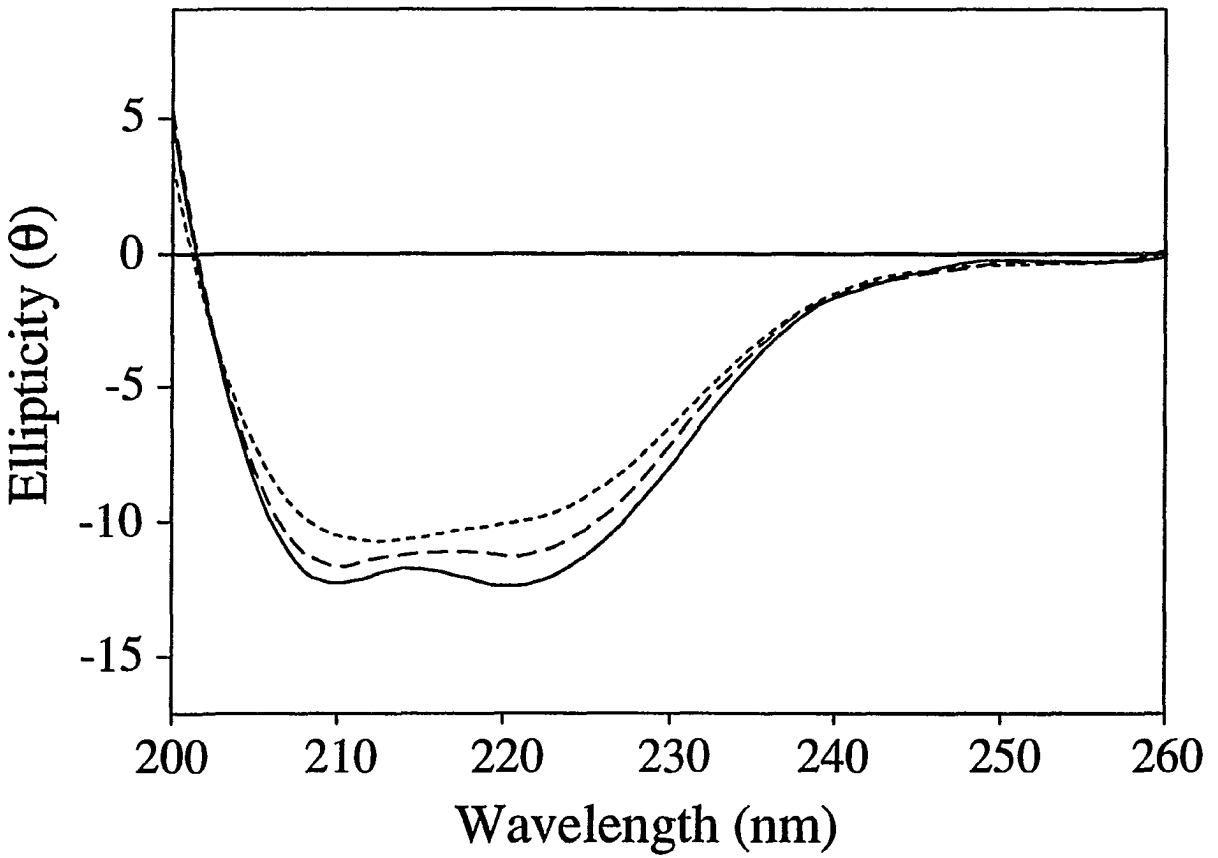

Fig. 1. CD spectra of mutant and wild-type adenylosuccinate synthetases. All spectra were normalized based on the concentrations of the protein used. CD spectra for G15V mutant AMPSase without substrate(solid line), with GTP(dashed line) or IMP(dotted line)

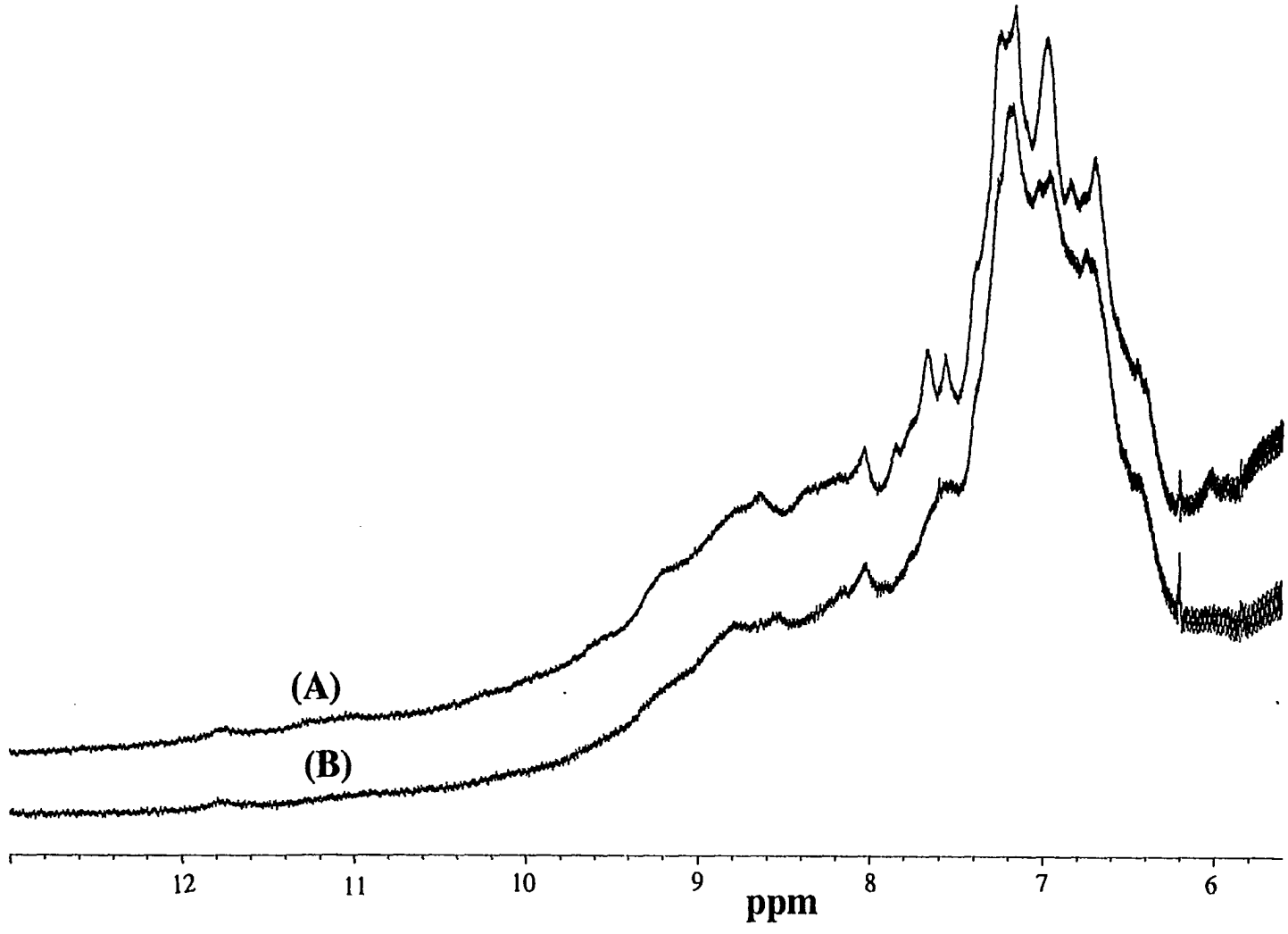

Fig. 2. Comparison of NMR spectra for wild-type (A) and the G15V mutant (B) AMPSase enzymes at pH ' = 7.7 in 20 mM Hepes. The chemical shifts (in parts per million) are referenced to the HDO peak which is assumed to be 4.80.

CHPATER II. REPLACEMENT OF Asp^{333} WITH Asn BY SITE-DIRECTED MUTAGENESIS CHANGES THE SUBSTRATE SPECIFICITY OF *ESCHERICHIA COLI* ADENYLOSUCCINATE SYNTHETASE FROM GTP TO XTP+

A paper published in *the Journal of Biological Chemistry*[1]

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ABSTRACT

The aspartate residue of the (N/T)KXD consensus sequence for GTP-binding proteins is present in the eight available sequences of adenylosuccinate synthetase. Reported here is a comprehensive analysis of the substrate specificity of mutant enzymes, where the conserved Asp^{333} of the synthetase from *Escherichia coli* is changed to asparagine, glutamate and glutamine by site-directed mutagenesis. The mutants D333N, D333E and

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D333Q generally show deceased k_{cat} values and increased K_m values for GTP. The decreased values of k_{cat} exhibited by the mutants indicate that the interactions between Asp^{333} and the guanine are relayed by some mechanism to the catalytic residues around the γ-phosphate of GTP, and that the energy provided by the interaction between Asp^{333} and the guanine moiety of GTP is utilized for rearrangement of the catalytic residues. The three mutants each have higher affinity for XTP and ITP than does the wild-type enzyme. In fact, the D333N mutant uses XTP more effectively than the wild-type enzyme employs GTP as a substrate. The side-chain of Asp^{333} forms hydrogen bonds with the N1 and the exocyclic amino group of the guanine base of GTP. In the D333N mutant, this interaction is probably replaced by hydrogen bonds between the amide side chain of Asn^{333} and N1 and the 2-oxo group of XTP. The D333Q mutant can use UTP as a substrate more effectively than the wild-type enzyme. The longer side chain of glutamine at residue 333 favors pyrimidine nucleotides over the purine nucleotides, GTP, XTP and ITP. These results demonstrate that Asp^{333} in the (N/T)KXD consensus sequence of adenylosuccinate synthetase from *E. coli* is a determinant for GTP-specificity.

INTRODUCTION

The catalytic power of enzymes is explained by the stabilization of the transition state which is brought about by utilizing the potential binding energy of substrates (1). Progress toward understanding the origins of substrate specificity is shown by several successes in altering the specificities of some enzymes by genetic engineering (2-5). This report describes varients of *Escherichia coli* adenylosuccinate synthetase (AMPSase)[a]

[a] The abbreviations used are: AMPSase, adenylosuccinate synthetase: DEAE HPLC, diethylaminoethyl high-pressure liquid chromatography: CD, circular dichroism: SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

[IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4] which have altered substrate specificities for purine and pyrimidine nucleoside triphosphates.

AMPSase (see ref. 6 for a review) catalyzes a reversible reaction with GTP, IMP and aspartate in the presence of Mg^{2+} to form adenylosuccinate, GDP and inorganic phosphate (P_i). This is the first reaction committed to the formation of AMP from IMP, using GTP as an energy source, and is believed to play an important role in the *de novo* pathway of purine nucleotide biosynthesis. No adenylosuccinate formation has been reported when ATP, CTP, UTP, or ITP replace GTP (7). In recent years, cDNA sequences encoding AMPSase have been cloned from several sources (8-15). It is reported (8) that their primary sequences contain the GTP-binding consensus sequences commonly identified in the GTPase superfamily (16). Alignment of the eight known amino acid sequences of AMPSases reveals that the GTP-binding sequences are well conserved. Among the sequences, Lys^{331} of *E. coli* AMPSase falls within the consensus box, (N/T)KXD, and was reported to be related to guanine base binding by our research group using site-directed mutagenesis (17). Also, X-ray diffraction studies (18) of crystalline AMPSase from *E. coli* suggests that the carboxylate group of Asp^{333} in the (N/T)KXD consensus sequence interacts with the C2 exocyclic amino group and a hydrogen atom of the N1 endo-nitrogen of the guanine base of GTP. A mutation of aspartate to asparagine at the corresponding residue in $p21^{ras}$ (19) reduced GTP binding affinity and a similar mutant (D138N) in elongation factor Tu (20) changed the base specificity from GTP to XTP. However, a comprehensive analysis of kinetics of the above mutants was not performed. This report below identifies position 333 of *E. coli* AMPSase as a key determinant in the recognition of nucleoside triphosphates, and that by modest changes in the side chain of residue 333, one can transform the wild-type, GTP-hydrolyzing enzyme into an even more proficient XTP-hydrolyzing enzyme. The modulation of substrate specificity observed here

by the mutation of the consensus aspartate may be broadly applicable to all GTP-binding proteins. The results below represent the first quantitative evaluation of the altered specificity of purine nucleotides in GTP-binding proteins.

EXPERIMENTAL PROCEDURES

Materials — *E. coli* strain XL1-Blue came from Stragene, a site-directed mutagenesis kit from Amersham, and restriction enzymes from Promega. GTP, UTP, ITP, XTP and Cibacron Blue 3GA were obtained from Sigma, and *pur A*$^-$ strain H1238 was a gift from Dr. B. Bachman (Genetic Center, Yale University).

Site-directed Mutagenesis — Recombinant DNA manipulation was performed using standard procedures (21). The primers in this study are shown in Table I. Mutagenesis and construction of the mutants cell lines were carried out according to the precedure previously described (17). The mutated plasmids were transformed into an *E. coli pur A*$^-$ strain (H1238), which does not produce AMPSase, in order to prevent mutant protein contamination by wild-type enzyme.

Preparation and Kinetics of Wild-Type and Mutant AMPSase — The mutant forms of AMPSase were purified by modification of the procedure described previously (22): Phenyl-sepharose, affinity chromatography, using a Ciba-cron blue column, and a DEAE HPLC column were used sequentially. The experimental details for these procedures are described elsewhere (17). Protein purities were analyzed by using SDS PAGE according to Laemmli (23). The concentration of the purified proteins in solution was determined using the extinction coefficient for wild-type AMPSase at 280 nm (ε_{280} = 67.85 $mM^{-1}cm^{-1}$), where the concentration refers to monomers. AMPSase activity was determined as described earlier (24), except that the absorbance change at 289 nm was monitered. The

concentrations of the stock solutions of the nucleotides were determined using their extinction coefficients at the proper wavelengths. For kinetic analysis of AMPSases, 3-100 μg/ml of enzyme was used depending on the activity of each mutant.

Circular Dichroism Spectroscopy — Circular Dichroism spectra were acquired at room temparature on an AVIV circular dichroism spectrometer, model 62DS, kindly supplied by Dr. Earl Stellwagen at the University of Iowa. The procedure used in this study was similar to that previously described (17). The protein concentration was 0.10-0.15mg/ml and the spectra were normalized for direct comparison.

UV Difference Spectra — At different times, UV spectra of the reaction mixtures were taken with proper amounts of the AMPSases and nucleotides using a Hewlett Packard Diode Array UV Spectrophotometer (model HP 8452A). Each spectrum was subtracted from that obtained at zero time.

RESULTS

Mutagenesis of AMPSase cDNA and Purification of the Mutants — As shown in Table II, the region including Asp^{333} in *E. coli* AMPSase is highly conserved in all eight AMPSases, where it corresponds to the (N/T)KXD region of GTP-binding proteins (16). The X-ray crystal stucture (18) of *E. coli* AMPSase implies that Asp^{333} may interact with the guanine base of GTP as shown in Fig. 1. Therefore, mutations at the conserved Asp^{333} are expected to alter the GTP binding specificity of AMPSase. In this study, Asp^{333} was changed to asparagine, glutamate and glutamine. The asparagine mutant (D333N) was prepared in order to disrupt the putative hydrogen bonds between Asp^{333} and the guanine base of GTP and form new hydrogen bonds with XTP, whereas glutamate and glutamine

(D333E and D333Q) were introduced at this position to evaluate the effects of side-chain length on substrate specificity.

The results of the purification of the mutants were similar to those of earlier studies (17). All AMPSase activities eluted at similar positions from a phenyl-sepharose column. The elution profiles from Ciba-cron Blue and DEAE HPLC columns were also almost identical for mutant and wild-type enzymes, which implies that the mutations in this study probably do not cause a global change in the protein structure. The mutants were not contaminated by any wild-type activity since the host cell line (H1238) does not contain wild-type AMPSase. The purities of the mutants were estimated as more than 95 % based on SDS PAGE (data not shown).

Circular Dichroism Spectroscopic Studies on the Mutant and Wild-type AMPSases — The CD spectra of wild-type and mutant AMPSases are superimposable (data not shown). Wild-type, D333N, D333E and D333Q AMPSases exhibit almost identical CD spectra which indicates that mutants of Asp^{333} have no major differences in secondary structure in spite of large differences in their kinetic parameters. Thus, the protein structures are not globally disrupted by the mutations prepared for this investigation.

Kinetic Analysis of AMPSase Mutants — Table III summarizes the kinetic parameters of the mutant and wild-type AMPSases using GTP, ITP, XTP, or UTP as substrates. Past studies (7) indicate that nucleotides other than GTP are not substrates for AMPSase. However, it was found that ITP can replace the natural substrate GTP, although its K_m value is extremely high (17.3 mM). Also, UTP and XTP can be used as substrates in the AMPSase reaction; however, their k_{cat} values are quite low (1.4 % and 3.7 %, respectively, relative to GTP). UV difference spectra in Fig. 2 show that the reaction catalyzed by the D333N mutant using XTP has an isosbestic point, $\Delta\lambda_{max}$ and $\Delta\lambda_{min}$ at 254 nm, 272 nm and 242 nm, respectively. These characteristics are the same as those observed

with the wild-type enzyme using GTP (25), which indicates that the product of the reaction catalyzed by the D333N mutant using XTP is adenylosuccinate. Likewise, for the other mutants, the product formed was adenylosuccinate (data not shown). The absorbance change at 289 nm observed with the wild-type enzyme using ITP proceeded for 1 hour after the reaction was initiated. The absorbance change indicated that more than 15 % of the ITP was consumed in the production of adenylosuccinate. Hence the AMPSase activity using ITP is not due to contamination of ITP by GTP, since the purity of ITP is more than 95 %. This is true as well for reactions using other nucleotides in this study (data not shown). In the case of ATP, wild-type AMPSase showed no detectable activity, even at a concentration higher than 10mM. As the only difference between ITP and GTP is the exocyclic amino group at C2, the interaction of this amino group with AMPSase may be critical to substrate recognition.

K_m values for GTP were 193 μM for D333N, 140 μM for D333E and 334 μM for D333Q. These results show that Asp^{333} of AMPSase is apparently involved in GTP-binding, provided one assumes that the rapid-equilibrium random Ter-Ter mechanism reported for wild-type AMPSase (24) is not changed in the mutants. However, The k_{cat} values for the D333N, D333E and D333Q mutants are 0.111/sec, 0.0189/sec and 0.0179/sec, respectively, using GTP as a substrate. Given that the X-ray results place the guanine-Asp^{333} interaction 20 Å from the site of hydrolysis, the results from kinetics imply that the Asp^{333}-guanine interaction may induce long range conformational changes, that are directly involved in the stabilization of the transition state. Since the kinetic mechanism of *E. coli* AMPSase is rapid equilibrium random, the value for k_{cat} is directly related to transition state stabilization. Hence the decrease in k_{cat} values for these mutants are likely due to the improper orientation of the substrate as a consequence of the unfavorable interaction of the 333 residue with the guanine base of GTP.

The D333N mutant uses XTP as an energy source more effectively than GTP, based on the k_{cat} and K_m values. The k_{cat} and K_m values for XTP are 1.14/sec and 33.1 μM, respectively (for GTP, 0.111/sec and 193 μM). Furthermore, the k_{cat} of the D333N mutant using XTP is 1.56 times higher than that of wild-type AMPSase using the natural substrate GTP. Comparison of the specificity constant (k_{cat}/K_m) for GTP and XTP is shown in Table IV. Compared to the wild-type AMPSase, the ratio of the specificity constants (XTP to GTP) in the D333N mutant is improved 2.86×10^4 times. Other mutants also show that GTP specificities are changed relative to XTP. This result clearly demonstrates that Asp^{333} is a major determinant of nucleoside triphosphate specificity for the AMPSase reaction. The high k_{cat}/K_m value for XTP exhibited by the D333N mutant probably stems from the strong hydrogen bonds, that can form between the xanthine base of XTP at the 2-oxo position and the side chain of Asn^{333} (Fig. 1).

For the D333Q mutant, UTP is the best substrate; the activity with GTP is only 18.3 % of that with UTP (Table III). Although its K_m is high (3.41 mM), the k_{cat} value of the D333Q mutant for UTP is 0.0978/sec, which is 8.81-fold higher than the k_{cat} value for the wild-type enzyme using UTP. This preference for UTP exhibited by the D333Q mutant probably is directly linked to the longer side chain and the amide group of the Gln residue relative to the Asp residue of the wild-type protein. The smaller ring size of UTP may be compensated to some extent by the longer side chain of Gln^{333} of the mutant. In fact, the ring system of UTP may interact with the side chain of Gln^{333} in a manner similar to the hydrogen bonding scheme for the D333N mutant and XTP (Fig. 1). The D333Q mutant can use ATP as a poor substrate (k_{cat}, 0.02/sec) in contrast to other mutant and wild-type AMPSases which show extremely low activity using ATP (k_{cat} less than 10^{-3}/sec, data not shown).

The D333E and D333Q mutants have longer side chains compared to the wild-type enzyme, resulting in dramatically reduced activities and increased K_m values for GTP, as shown in Table III. However, the K_m values for XTP of these mutants are similar to that of the D333N mutant. The relatively low values of K_m for XTP in the case of the D333E and D333Q mutants are not readily explained in the absence of detailed structural information regarding their substrate enzyme complexes. These results show that the side-chain length and its functional group at position 333 of AMPSase are optimized for GTP in the wild-type enzyme.

DISCUSSION

Replacement of Asp^{333} by Asn in a consensus GTP-binding region in *E. coli* AMPSase produces an enzyme which proficiently uses XTP as a substrate at the expense of a much reduced activity toward the natural substrate GTP. Another mutant, D333Q exhibits significantly improved activities using UTP and ATP as substrates relative to the wild-type enzyme. These results are the first quantitative evaluation of the altered specificity of purine nucleotides in GTP-binding proteins, including metabolic enzymes that use GTP as a specific energy source.

As demonstrated in this study, Asp^{333} is a determinant for GTP specificity of *E. coli* AMPSase. The Asn mutants of corresponding aspartate residues have been reported in $p21^{ras}$ (19) and elongation factor Tu (20). In $p21^{ras}$, the mutation (D119N) reduced GTP binding affinities by 100-fold. The corresponding mutation (D138N) in the elongation factor altered its substrate specificity from GTP to XTP, but a quantitative analysis of substrate specificities was not performed. These results suggest that the conserved aspartate in the (N/T)KXD consensus sequence is an element used by the GTP-binding proteins in

the recognition of purine nucleotides. The fact that the interactions of the (N/T)KXD box with GTP in the AMPSase structure are similar to that of $p21^{ras}$ (18) suggests that the same type of mutational effects described in this report would be expected with other GTP-binding proteins.

The relative values of k_{cat}/K_m for GTP for the position 333 mutants of AMPSase (Table III) suggest that this residue may contribute to catalysis as well to the binding of GTP. As mentioned earlier, the interaction between the Asp^{333} and the guanine moiety of GTP may induce long-range interactions with other catalytic residues of the enzyme. Two general mechanisms for long-range interactions are conceivable: i) the Asp^{333}-GTP interaction insures the correct orientation and position of the γ-phosphate, ii) the interaction of Asp^{333} with GTP induces reorganization of other catalytic machinery (for example, the P-loop of this enzyme) (26), whereas mutants, deficient in the interaction, provide insufficient energy for transition-state stabilization. In fact, both mechanisms may be at work in the case of the synthetase, as the proper binding of the γ-phosphate of GTP may provide the energy for the reorganization of the active site.

The K_m values for ITP decrease as one goes down Table III. The opposite tendency is evident in the K_m values for GTP. Therefore, it seems that the unfavorable interactions between guanine and position 333 of the mutants becomes more favorable with ITP. Recently, a Raman spectroscopic study (27) of GTP-binding proteins revealed that the hydrogen bonds between the proteins and the 6-keto group of the guanine base were stronger for GDP in EF-Tu than in $p21^{ras}$, but that this was reversed for bound IDP. These results suggested that the removal of the 2-amino group from GDP influenced the interactions at the 6-oxo group of purine ring. Likewise, the anticorrelation in the K_m values for GTP and ITP for the set of mutants of this study may stem from local structural changes around position 333 in *E. coli* AMPSase. Crystal structures of AMPSases with

nucleotides including XTP may provide information regarding these conformational changes in the synthetase.

Previously, our labolatory has reported that two mutants at Lys^{331} (K331R and K331L) have different K_m values for GTP, but similar k_{cat} values relative to the wild-type enzyme (17). In contrast to the mutants at Lys^{331}, the Asp^{333} mutants have greatly reduced activity as well as reduced affinity for GTP. Apparently, the interaction energy of Lys^{331} with GTP is used only for binding, but that of Asp^{333} is used for both binding and catalysis. These results imply that only specific types of interactions between substrates and enzymes allow utilization of substrate binding energy for catalysis and specificity.

Even though the mutants in this work have low AMPSase activities with GTP, they grow very well without any added adenine in the medium. In fact, the intracellular concentration of GTP in *E. coli* ranges from about 1 mM (28) to 4 mM (29), a level at which all of the mutants in this report will have their maximum activities. Considering that these mutant cell lines overexpress at least 40-fold (22), the D333Q mutant, despite its low AMPSase activity (2.4 % of the wild-type AMPSase), could provide enough adenylosuccinate to meet the physiological needs of the bacteria for the synthesis of AMP.

The transformation of AMPSase from a GTP-binding to an ATP-binding enzyme must go beyond mutations at position 333. The ATP molecule has a 6-amino group instead of the 6-oxo group of GTP. Thus, residues that hydrogen bond to the 6-oxo group of GTP must change from proton donors to proton acceptors in an interaction with ATP. In the case of *E. coli* thymidylate synthase (5), for instance, a mutant (N177D) changed the nucleotide specificity from dUTP to dCTP, whereas the wild-type enzyme used dUTP as a substrate exclusively. The difference between dUTP and dCTP is similar to that between GTP and ATP. In this study, the D333Q mutant used ATP as a substrate, although it exhibits only 2.5 % of the activity of the wild-type enzyme using GTP. Also, the same

mutant can use UTP as a substrate with activity 13.3 % of the wild-type enzyme with GTP(Table III). However, it may be possible to engineer AMPSase into a proficient ATP or UTP-using enzyme by the modification of residues surrounding the 6-oxo group of GTP or 4-oxo group of UTP, together with mutations at Asp^{333}. Those studies are currently proceeding in our laboratory in order to identify additional determinants of substrate specificity of AMPSase.

In summary, we have shown that XTP is a better substrate for D333N AMPSase, than is GTP for the wild-type enzyme, using k_{cat} as a criterion. In addition, we report that Asp^{333} of (N/T)KXD in *E. coli* AMPSase contributes directly to GTP-binding as well as to catalysis through long-range interactions. By mutating this residue to Asn, Glu and Gln, we have generated AMPSases with greatly altered specificity toward nucleoside triphosphates. Particularly, the D333N mutant specifically uses XTP and the D333Q mutant favors UTP as an energy source. In contrast to Lys^{331} mutants previously reported by our labolatory (17), a portion of the binding energy derived from the interaction of Asp^{333} with guanine of GTP may be used in reorganizing catalytic residues.

Note Added in Proof - Since completion of this research, Weijland and Parmeggiani (1994, *Trands Biochem. Sci.* **19,** 188-193) published an article in which they presented hydrogen bond interactions of XTP at the GTP binding site of an elongation factor Tu mutant (D138N), that is identical to that described in Fig. 1. They attributed the interaction beween Asn^{138} and XTP to Hwang and Miller's (1987, *J. Biol. Chem.* **262,** 13081-13085); however, these investigators did not suggest the formation of a 6-membered hydrogen network beween Asn^{138} and XTP.

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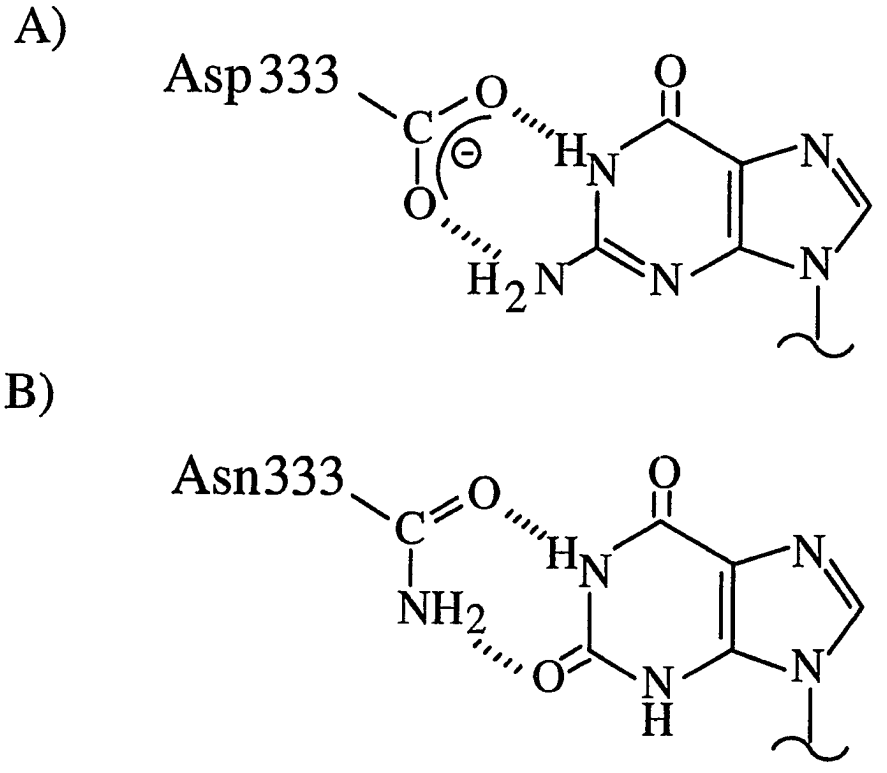

Fig. 1. A proposed model for the interations between NTP and the side chain of the 333 position of *E. coli* AMPSase; (a) GTP and Asp^{333}, (b) XTP and Asn^{333}

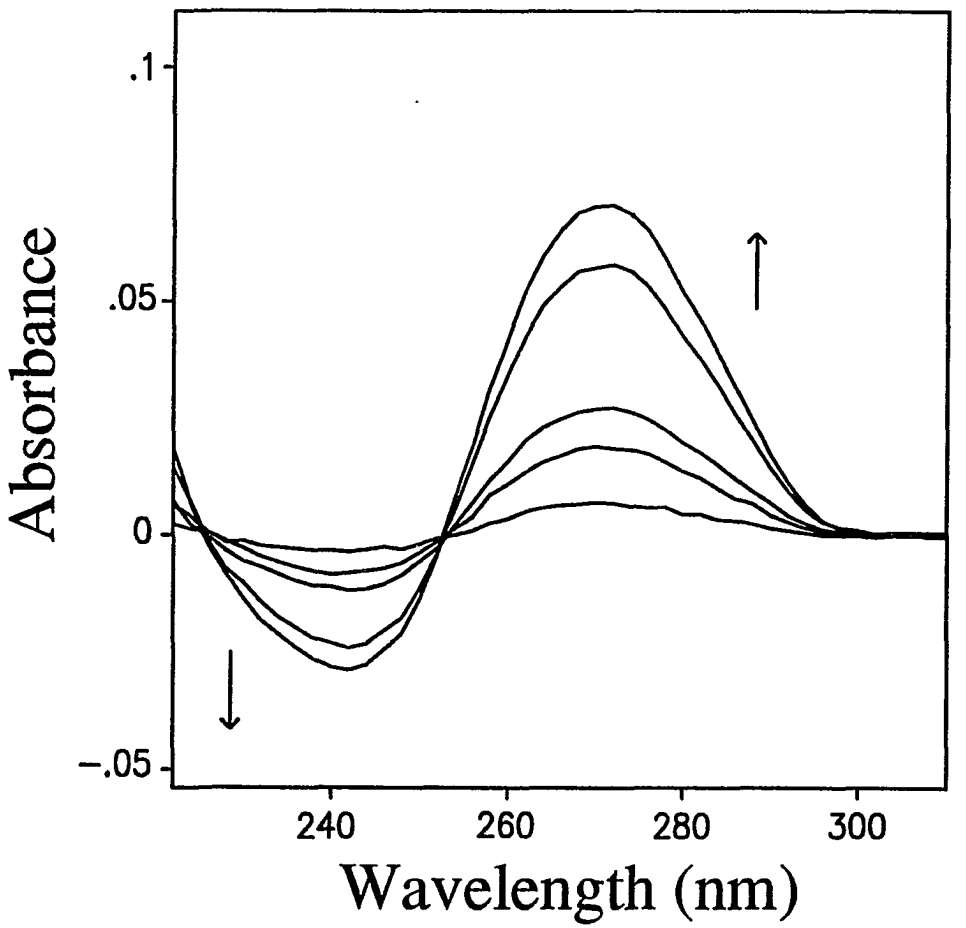

Fig. 2. UV-Vis difference spectra of the AMPSase reaction of the D333N mutant with XTP at different times. The components in the reaction mixture were: 0.05 mM IMP, 5 mM Asp, 5 mM $MgCl_2$, 0.05 mM XTP, 2 μg of D333N mutant and 10 mM of Hepes buffer (pH = 7.7). Arrows indicate absorbance changes with increasing time.

Table I Oligonucleotides Used in Site-Directed Mutagenesis.[a]

Mutants	Sequence of Primers
D333N	5'-TCCAGAACGT**T**CAGTTTAGT
D333E	5'-CCAGAAC**C**TCCAGTTTAGTC
D333Q	5'-CCAGAAC**CTG**CAGTTTAGTC

[a] The mismatched bases are shown in boldface.

Table II Comparison of Putative Guanine Recognition Elements in Known Sequences of AMPSase.

Species	Sequence	Ref.
Human Liver	LTKL**D**IL	12
Mouse Muscle (acidic)	LTKL**D**IL	9
Dictyostelium dicoidium	LTKL**D**VL	10
Schizosaccharomyces pombe	LTKL**D**IL	13
Escherichia coli	LTKL**D**VL	8
Thiobacillus ferrooxidans	ITKL**D**VL	15
Bacillus subtilis	LNSI**D**VL	11
Mouse Muscle (basic)	LTKL**D**IL	14

The conserved Asp residues are in boldface with larger font.

Table III Comparison of the Wild-Type and Asp^{333} Mutant AMPSases

Proteins	GTP		UTP		ITP		XTP	
	k_{cat} (/sec)	$K_{m,GTP}$(μM)	k_{cat} (/sec)	$K_{m,UTP}$(mM)	k_{cat} (/sec)	$K_{m,ITP}$(mM)	k_{cat}(/sec)	$K_{m,XTP}$(μM)
Wild-Type	0.733 ± 0.096	22.2 ± 5.4	0.0111 ± 0.0009	2.06 ± 0.40	0.628*± 0.17	17.3*± 6.34	0.0268 ± 0.0016	388 ± 63
D333N	0.111 ± 0.003	193 ± 10.9	0.0210 ± 0.0015	1.27 ± 0.18	0.0622 ± 0.0067	3.29 ± 0.64	1.14 ± 0.07	33.1± 5.2
D333E	0.0189 ± 0.0029	140 ± 44	0.011± 0.003	4.82 ± 2.59	0.0743 ± 0.0091	2.87 ± 0.69	0.0523 ± 0.0017	28.5 ± 3.5
D333Q	0.0179 ± 0.0015	334 ± 73	0.0978 ± 0.0192	3.41 ± 0.97	0.0439 ± 0.0031	1.07 ± 0.23	0.0599 ± 0.0044	54.7 ± 8.3

A typical enzyme assay solution contained 450 μM IMP, 5 mM Asp, 40 mM Hepes (pH 7.7), 5 mM $MgCl_2$ and appropriate amount of AMPSases.

* 15 mM $MgCl_2$ was used.

Table IV Comparison of specificity constants of the AMPSases for GTP and XTP

	Specificity Constant for GTP($k_{cat}/K_{m,GTP}$)	Specificity Constant for XTP($k_{cat}/K_{m,XTP}$)	Relative Substrate Preference (RSP) XTP($k_{cat}/K_{m,XTP}$) / GTP($k_{cat}/K_{m,GTP}$)	
Wild-Type	3.30×10^{-2}	6.91×10^{-5}	2.09×10^{-3}	(1)*
D333N	5.75×10^{-4}	3.44×10^{-2}	59.8	(2.86×10^{4})*
D333E	1.35×10^{-4}	1.84×10^{-3}	13.6	(6.51×10^{3})*
D333Q	5.36×10^{-5}	1.10×10^{-3}	20.5	(9.82×10^{3})*

* Relative values ; RSP(D333N)/RSP(Wild-Type)

CHAPTER III. IDENTIFICATION OF AN ESSENTIAL SECOND METAL ION IN THE REACTION MECHANISM OF *ESCHERICHIA COLI* ADENYLOSUCCINATE SYNTHETASE[+]

A paper published in *the Journal of Biological Chemistry*[1]

Chulhun Kang[2] and Herbert J. Fromm[2, 3]

ABSTRACTS

This study reports that two Mg^{2+} ions are required for *Escherichia coli* adenylosuccinate synthetase activity. The first metal ion is presumably coordinated with β- and γ-phosphoryl groups of GTP to provide an electron sink, and the second one seems to interact with aspartate in the enzyme active site. Regarding the latter metal ion, kinetic studies show that aspartate and the second Mg^{2+} ion bind to the enzyme active site randomly with a k_{cat} value of 1.47/sec and with K_m values for aspartate and Mg^{2+} of 225and 114 μM, respectively. The dissociation constants for aspartate and Mg^{2+} of the enzyme·GTP·IMP·(aspartate or Mg^{2+}) complex are 79.2 and 40.0 μM, respectively.

[+] This research was supported in part by national Institutes of Health, United States Public Health Service, Research Grant NS 10546 and National Science Foundation Grant MCB-9218763. This is Journal Paper 16116 of the Iowa Agriculture and Home economics Experiments Station, Ames, IA: Project 2575.

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However, variable amounts of aspartate or Mg^{2+} did not show any significant changes in the K_m values for GTP and IMP. Kinetic studies using Mn^{2+} and Ca^{2+} ions indicate that the k_{cat} values(0.930 and 0.235 /sec, respectively) were slightly decreased compared to the value obtained using Mg^{2+}; however, the K_m values for aspartate and GTP in the presence of Mn^{2+} and Ca^{2+} were significantly decreased compared to those obtained using Mg^{2+} ion(4.5 and 4.6 times for Mn^{2+} ion, and 5.6 and 5.8 times for Ca^{2+} ion, respectively). On the other hand, the K_m values for IMP were not significantly changed(1.9 and 1.8 times for Mn^{2+} and Ca^{2+} ions, respectively). Taken together, these kinetic results imply that aspartate may interact with Mg^{2+} to form a Mg·aspartate complex in the enzyme active site. An inhibition study of the enzyme with $ZnCl_2$ (its K_i value is 29 nM) also suggested that Zn^{2+} competes with aspartate as well as Mg^{2+}, implying that Zn^{2+} might form a complex with aspartate in the active site. On the basis of these results, it is suggested that Mg·aspartate complex formation in the active site of adenylosuccinate synthetase may be important in activation of the protonated amino group of aspartate, enhancement of the enzyme's binding affinity, and its specificity for aspartate.

INTRODUCTION

The production of nucleotides is a mandatory function of living cells. The broad outlines of *de novo* purine nucleotide synthesis have been known since the late 1950s when Buchanan and coworkers (1) outlined the 10 reactions necessary to convert phosphoribosyl pyrophosphate to IMP, which is then utilized for AMP and GMP synthesis. Since then, research has been focused on individual enzymes and their relationship to other members of the pathway.

Adenylosuccinate synthetase[a] (AMPSase) (see ref. 2 for review) catalyzes the following reversible reaction in the presence of Mg^{2+} ion, which participates in the reaction as a GTP·Mg complex:

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

This reaction is the first committed step in the formation of AMP from IMP, using GTP as a specific energy source, and is believed to play an important role in the *de novo* pathway of purine nucleotide biosynthesis.

In recent years, cDNA sequences encoding AMPSase have been cloned from several sources (3-10). Alignment of the known amino acid sequences reveals that a high degree of homology is found throughout the sequences except in the regions of subunit contacts and the C-terminal region. Comparison of the amino acid sequences of AMPSases with the GTP-binding proteins showed that the AMPSases share several consensus sequences with GTP-binding proteins. X-ray diffraction studies (11) of crystalline AMPSase from *Escherichia. coli* revealed that, although the folding patterns of Ha-Ras p21 protein and AMPSase are significantly different, the elements bound to the guanine and phosphate moieties of GTP are similar in both proteins. The aspartate residue at position 333 of *E. coli* AMPSase is responsible for the exquisite substrate specificity of GTP of the protein. Mutations at this position changed the substrate specificity of AMPSase from GTP to other nucleotides (12). Also, Lys^{331} of the enzyme was reported to be responsible for GTP binding to the protein (13).

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[a] The abbreviations used are: AMPSase, adenylosuccinate synthetase: HPLC, high-pressure liquid chromatography: SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis: SAICAR synthetase, 5-aminoimidazole-4-N-succinocarboxamide ribonucleotide synthetase.

6-phosphoryl IMP has been suggested as an intermediate in the reaction mechanism of AMPSase by Lieberman in 1956 (14) and by Fromm in 1958 (15). Kinetic studies of *E. coli* AMPSase demonstrated that the substrates bind to the enzyme active sites randomly (16), and an isotope exchange experiment with mammalian AMPSase suggested that aspartate preferred binding to the E·GTP·IMP complex rather than to the free enzyme (17). The amino group of aspartate performs a nucleophilic substitution at the 6-carbon of 6-phosphoryl IMP in a highly specific manner in which only NH_2OH is an alternative substrate for aspartate with significant activity (14). There is an absolute requirement for divalent metal ions by AMPSase, and one metal ion was postulated to stabilize developing partial negative charges on the γ-phosphoryl group of GTP during nucleophilic substitution at the 6-oxo group of IMP. Two reports seem to raise an interesting question on how many metal ions are necessary in the AMPSase reaction mechanism. In Lieberman's work (14) regarding the metal ion requirement, although 0.14 mM GTP was used, the apparent K_m for total $MgCl_2$ was approximately 0.8 mM, which gives an unusally high value for the dissociation constant for Mg^{2+} of the GTP·Mg complex. Also, addition of Ca^{2+}, Mn^{2+}, or Zn^{2+} at concentrations 1/100 of the Mg^{2+} level always caused inhibition (2). Although these metal ions were suggested to perform a regulatory role, only one metal ion, Mg^{2+}, has been considered to be important in the reaction mechanism of AMPSase (2).

Because, generally, the protonated amino group of aspartate can be considered as a very weak nucleophile, some base(s) can be expected to be present in the active site of AMPSase to remove the proton from aspartate. In this report, we studied the stoichiometry of the divalent metal ion required in the enzyme reaction and demonstrate that two divalent metal ions are necessary for enzyme activity. One of these metal ions functions by activating aspartate as a strong nucleophile at the active site. Also, to characterize the

detailed role of the metal ion, kinetic studies with other divalent metal ions including Zn^{2+} were performed, and the biological significance of these studies is discussed.

EXPERIMENTAL PROCEDURES

Materials — Ultra pure hydrate forms of $MgCl_2$ (99.999 %), $MnCl_2$ (99.999 %), $CaCl_2$ (99.99 %), and $ZnCl_2$ (99.995 %) were purchased from Aldrich, and GTP, IMP, aspartate, and a chelating resin were obtained from Sigma Chemical Co. A *pur A*$^-$ strain (H1238) was a gift from Dr. B. Bachman (Genetic Center, Yale University).

Preparation of E. coli AMPSase — Phenyl-sepharose, affinity chromatography, using a Cibacron blue column, and a DEAE HPLC column were used sequentially to purify *E. coli* AMPSase from an overexpressed cell line of H1238. The experimental details for these procedures were described elsewhere (18). The purity of the prepared protein was more than 95 % as judged by SDS PAGE according to Laemmli (19). The purified AMPSase was demetalized by using a chelating resin according to the literature (20). The concentration of the purified protein in solution was determined by using the extinction coefficient for wild-type AMPSase at 280 nm (ε_{280} = 67.85 $mM^{-1}cm^{-1}$), where the concentration refers to monomers.

Kinetic Analysis of Adenylosuccinate Synthetase — AMPSase activity was determined as described earlier (16), except that the absorbance change at 22 °C was monitored at 290 nm and converted to that at 280 nm using the ratio of the absorbance changes at 290 and 280 nm (0.30) unless stated otherwise. The concentrations of the stock solutions of the nucleotides were determined by using their extinction coefficients at the proper wavelengths. For kinetic analysis of AMPSase, 1.0 μg/ml of enzyme was used. GTP and IMP were kept at approximately 10 times their K_m values. The assay solution was

incubated with enzyme, GTP, IMP and Asp at 22 °C for 20 min and, finally, proper amounts of $MgCl_2$ were added before the absorbance changes were measured. The kinetic parameters in Table II were deduced by linear regressional analysis using a MINITAB computer program (21).

Kinetic experiments using Mg^{2+}, Ca^{2+} and Mn^{2+} ions were performed in a manner similar to that described elsewhere (13). In Zn^{2+} inhibition studies, other components including Zn^{2+} ion in the assay solution were mixed and incubated for 20 minutes, and $MgCl_2$ or aspartate was finally added to the reaction mixtures for the experiment in which Mg^{2+} or aspartate was varied. The kinetic experiments for GTP and IMP in the presence of Zn^{2+} ion were similarly performed. The detailed experimental conditions are described in the figure legands. Except for the kinetic results shown in Table II, all of the V_{max} and K_m values in this report were determined by using a nonlinear regression computer program (ENZFITTER) (22).

Calculation of free substrate concentrations in the reaction mixture was done by using a modified iteration program based on O'Sullivan and Smithers' algorithm (23). In these calculations, stability constants for various complexes were obtained from the literature, and modified to the values at pH 7.7 which is used in this study (23-26). The stability constants used in this study are 3.2/M, 73000/M, and 70/M for aspartate·Mg, GTP·Mg, and IMP·Mg, respectively, and 7480/M, $1.12/M^2$, 100,000/M, and 400/M for aspartate·Zn, $(aspartate)_2$·Zn, GTP·Zn, and IMP·Zn, respectively, at pH 7.7. Metal ion binding ability to Hepes buffer was neglected.

RESULTS

Mg^{2+} Ion Requirement in the AMPSase Reaction — One of the reaction mechanisms suggested for AMPSase involves a 6-phosphoryl IMP intermediate (14, 15). In this mechanism, no partial reaction occurs until all substrates bind at the enzyme's active site. Then, the 6-oxo group of IMP, a nucleophile, attacks the γ-phosphoryl group of GTP, resulting in 6-phosphoryl IMP and GDP. Finally, the amino group of aspartate performs another nucleophilic substitution on the 6-carbon of IMP, which is esterified to phosphate of the intermediate, forming adenylosuccinate and free phosphate. The divalent metal ion is considered to be coordinated with the β- and γ-phosphate groups of GTP, and stabilization of developing partial negative charges on the phosphates occurs. However, because the apparent K_m value for Mg^{2+} was found to be unusally high, as reported in Lieberman's work (14), it may be possible that AMPSase requires another metal ion besides the divalent metal ion associated with GTP. Therefore, in this study, experiments were conducted to investigate the metal ions in *E. coli* AMPSase. Indeed, as shown in Fig. 1, the AMPSase activity increases sigmoidally as the total $MgCl_2$ concentration increases. A Hill-type plot (shown in the inset of Fig. 1) of these data shows that two Mg^{2+} ions are necessary for expression of AMPSase activity. Hence, it can be assumed that a relatively weak metal complex is necessary in addition to the metal associated with GTP. On the basis of these results, it was possible to deduce a kinetic model, as shown in Scheme I, which is a modified version of rapid equiliblium random Ter-Ter kinetic model (16).

To characterize the possible roles of the second metal ion in the AMPSase reaction, the activity was measured at various concentrations of substrates and Mg^{2+}. The aspartate-dependency of the enzyme activity at variable amounts of $MgCl_2$ is shown in Fig. 2. In Fig. 2, although at least 95 % of GTP should exist as the GTP·Mg complex throughout the

experiment as judged by its known stability constant (73000/M), aspartate and Mg^{2+} ion increase the enzyme activity synergistically. Table I demonstrates that as the aspartate concentration increases, the $K_{m,app}$ and $V_{max,app}$ values for Mg^{2+} gradually increase and reach limiting values. The $K_{m,app}$ and $V_{max,app}$ values for aspartate also show a similar result when the concentration of Mg^{2+} increases (data not shown). Based upon these results, a kinetic model shown in Scheme I can be established. In order to determine the kinetic parameters for aspartate and Mg^{2+} ion, kinetic studies varying $MgCl_2$ or aspartate were performed at saturating levels of GTP and IMP concentrations which allow GTP- or IMP-related terms to be ignored. Thus, the kinetic equation for Mg^{2+} and aspartate was simplified when the concentration of Mg^{2+} ion was varied at a fixed level of aspartate to give the following equation:

$$\frac{V_{max}}{V} = 1 + \frac{K_{d,Asp} K_{m,Mg}}{[Asp][Mg^{2+}]} + \frac{K_{m,Mg}}{[Mg^{2+}]} + \frac{K_{m,Asp}}{[Asp]} \qquad \text{(Eq. 1)}$$

$$V_{max,app} = V_{max}\left(1 + \frac{K_{m,Asp}}{[Asp]}\right)^{-1} \quad \text{and} \quad \frac{K_{m,app}}{V_{max,app}} = \frac{K_{m,Mg}}{V_{max}} \cdot \left(\frac{K_{d,Asp}}{[Asp]} + 1\right)$$

$$K_{d,Mg} \cdot K_{m,Asp} = K_{d,Asp} \cdot K_{m,Mg}$$

$K_{d,Mg}$: Dissociation constant of Mg^{2+} from E·GTP·IMP·Mg

$K_{d,Asp}$: Dissociation constant of aspartate from E·GTP·IMP·aspartate

$K_{m,Mg}$ and $K_{m,Asp}$: Michaelis constants for Mg^{2+} and aspartate, respectively

The double recoprocal plots for initial velocity *versus* the concentrations of Mg^{2+} ion and aspartate are shown in Fig. 3(A) and (B), respectively. Based upon the model described in Scheme I and Equation 1, a statistical analysis of the data provided kinetic parameters for Mg^{2+} and aspartate which are summarized in Table II. K_m values for Mg^{2+} ion and aspartate are 114 and 225 μM, respectively, but the dissociation constants ($K_{d,Mg}$ and

$K_{d,Asp}$) of Mg^{2+} ion and aspartate for the E·GTP·IMP complex are 40.0 and 79.2 μM, respectively. These values are approximately 3 times smaller than the corresponding K_m values. Comparison of the dissociation constants and K_m values indicate that there are unfavorable interactions between aspartate and Mg^{2+} in the E·GTP·IMP·Mg·aspartate complex compared to the interactions occuring when either aspartate or Mg^{2+} ion binds to the E·GTP·IMP complex. These findings when taken with the fact that many complexes are known to form between amino acids and metal ions (25), make it likely that the second matal ion forms a complex with aspartate in the active site of AMPSase and thus facilitates the nucleophilic substitution of the amino group of aspartate at the 6-carbon of the 6-phosphoryl IMP. This mechanism is depicted in Fig. 4. On the basis of this suggestion, aspartate specificity of AMPSase including the dianion requirement for aspartate (27) can be explained, although it remains to be shown which residues interact with the divalent metal ion and the two carboxylate moieties of aspartate.

Metal Ion Selectivity in AMPSase Reaction — Based upon the model shown in Fig. 4, it is interesting to ask whether or not other divalent metal ions will have significant effects on binding of aspartate as well as GTP. To pursue the answer, kinetic experiments were carried out for each substrate by using Mg^{2+}, Ca^{2+} and Mn^{2+} ions where the concentration of other substrates were held at their saturation levels. A comparison of the kinetic parameters with Mg^{2+}, Ca^{2+} and Mn^{2+} ions are shown in Table III. The k_{cat} values are 1.26, 0.235 and 0.930/sec for Mg^{2+}, Ca^{2+} and Mn^{2+} ions, respectively. Although the K_m values for IMP using Ca^{2+} and Mn^{2+} ions are slightly reduced (approximately 1.8-fold for both) compared to that with Mg^{2+} ion, the K_m values for GTP and aspartate are changed significantly compared to those using Mg^{2+} ion (approximately 3 to 6-fold decreases for both substrates using either metal ion). These results suggest that the divalent metal ions are involved in aspartate-binding as well as GTP binding in the reaction

mechanism of AMPSase. This suggestion is consistent with the model shown in Fig. 4.

Inhibition Studies Using Zn^{2+} Ion — Zn^{2+} ion has been shown to be a potent inhibitor of AMPSase even in the presence of a 100-times excess amount of Mg^{2+} (2). This finding cannot be explained solely on the formation of GTP·metal ion complex in the enzyme active site because the stability constants for Mg·GTP and Zn·GTP are comparable (26). Hence, an attempt to elucidate the Zn^{2+} ion inhibition mechanism was undertaken by using the model shown in Scheme I. Table IV depicts the kinetic parameters of *E. coli* AMPSase in the presence of Zn^{2+} ion. The results show that the inhibition by Zn^{2+} ion is competitive against both aspartate and Mg^{2+}. A detailed data analysis was not attempted because the Zn^{2+} ion concentration could not be held constant when aspartate is varied. However, it was possible to deduce a K_i value for Zn^{2+} from the kinetic paremeters when Mg^{2+} is varied. With a kinetic model where Zn^{2+} ion competed with Mg^{2+} in Scheme I, the apparent K_i value for Zn^{2+} was estimated to be 29 nM. This value is 3.93×10^3 times lower than the $K_{m,Mg}$ value (114 μM), which is close to the reported ratio of the stability constants for aspartate·Zn^{2+} to aspartate·Mg^{2+}, which is approximately 3.9×10^3 (25). These results strongly suggest that aspartate may be complexed with divalent metal ions in the AMPSase active site. This proposal is further supported in that most of the strong metal ion inhibitors of AMPSase such as Cu^{2+}, Cd^{2+}, and Pb^{2+} are good chelators of aspartate as well (24). Parallel experiments investigating the effects of Zn^{2+} ion on GTP or IMP binding properties revealed that the K_m values for both substrates exhibit minor variations, but the V_{max} values decreased in the presence of Zn^{2+} as shown in Table IV. These results are consistent with the suggestion that the aspartate·Zn complex forms in the active site of AMPSase and inhibits the enzyme.

DISCUSSION

The studies presented in this report were undertaken in an attempt to understand the role of divalent metal ions in the AMPSase reaction. We demonstrated that the enzyme needs two divalent metal ions for activity. One is associated with the Mg·GTP complex, and the second metal ion may be complexed with aspartate to facilitate its nucleophilic attack on 6-phosphoryl IMP. These findings provide new insights into the reaction mechanism as well as the molecular basis of Zn^{2+} ion inhibition of AMPSase.

Among the enzymes using aspartate as a substrate, AMPSase has a relatively high affinity for this substrate. For instance, the dissociation constants for aspartate with other enzymes such as aspartase (28), aspartate aminotransferase (29), or aspartate transcarbamylase (30) are 4.3 mM, 4.0 mM, and 12.2 mM, respectively. The dissociation constant for aspartate was reported as 1.0 mM for *Azotobacter vinelandii* AMPSase (31) in the absence of any ligand in the active site, which is close to the K_m values of the enzymes mentioned. However, as shown in Table II, the affinity of AMPSase for aspartate becomes enhanced by the presence of GTP and IMP ($K_{d,Asp}$ is 79.2 μM) and is approximately 100-fold greater compared with those of the other enzymes cited. This strong affinity of AMPSase for aspartate may provide a driving force for the deprotonation of the amino group of aspartate by metal ion. At physiological pH (7.7), the amino group of aspartate exists in the protonated form. At the active site, however, due to complexation between Mg^{2+} and aspartate, the equiliblium may be shifted to the deprotonated form of the amino group, which is a good nucleophile. This type of activation of a nucleophile can be seen in the case of adenosine deaminase, where a hydroxide ion is generated from a water molecule coordinated with Zn^{2+} ion and acts as a nucleophile (32). Also, the formation of a Mg·aspartate complex in the AMPSase active site may provide a rationale for aspartate

specificity because the formation of metal complexes by amino acids is known to be highly stereoselective (33). On the basis of the crystal structure of the Mg·aspartate complex (34), the two carboxyl groups of aspartate, which were reported to be important in the AMPSase reaction (27), coordinate with Mg^{2+}, but the interactions of glutamate with metal ions are different from those of aspartate, presumably due to the additional methylene group in glutamate (24). Likewise, compared with other amino acids, aspartate can form unique complexes with metal ions, which may provide the aspartate specificity of the AMPSase reaction. Another advantage of the formation of the Mg·aspartate complex at the active site may be enhancement of nucleophilicity of the amino group of aspartate.

It may be possible to suggest that a metal-binding site is located somewhere in the protein outside the active site in order to explain the critical relationship between aspartate and the second metal ion demonstrated in this study. This suggestion requires the assumption that long-range interations occur between those two sites in order to provide an explanation for the finding that metal-binding controls the properties of aspartate-binding and its reactivity. However, based upon this model, it is difficult to explain the exquisite specificity for aspartate since glutamate or other analogs for aspartate failed to show any activity with AMPSase.

The reaction mechanism of AMPSase requires two nuceophilic substitutions that are nucleophilic attacks on the γ-phosphoryl group of GTP by the 6-oxo group of IMP and on the C-6 of phosphoryl-IMP by the amino group of aspartate. These two nucleophilic substitutions are presumably facilitated by catalytic bases to remove protons from the putative nucleophiles. For the former nucleophilic attack, Asp^{13}, a residue belonging to the P-loop, is suggested to play the role of a base to increase the nuceophilicity of 6-oxo group of IMP by removing the proton on the purine ring system of IMP (Richard B. Honzatko, personal communication). For the latter, as shown in Fig. 4, Mg^{2+} ion may perform the

role of the base. However, to depict the exquisite aspartate specificity in the AMPSase reaction, interactions between the protein and aspartate other than the metal complexation are required. The most plausible candidate may be interaction of the β-carboxyl group of aspartate with positively charged residues in the active site. Currently, we know that a mutation at Arg^{131} of AMPSase causes a significantly decreased affinity for aspartate. Characterization of the detailed effects of site-specific mutaions at Arg^{131} on the specificity of aspartate and on catalysis is currently underway.

It is of interest to note that 5-aminoimidazole-4-N-succinocarboxamide ribonucleotide synthetase (SAICAR synthetase), which is involved in *de novo* purine nucleotide synthesis, is similar to AMPSase in many respects, including the substrates, reaction mechanisms (1) and amino acid sequence segments. SAICAR synthetase uses ATP and aspartate for the formation of SAICAR from carboxyaminoimidazole ribotide (CAIR), which is similar to IMP, whereas AMPSase forms adenylosuccinate from IMP using GTP and aspartate. Both enzymes utilize Mg^{2+} ions as the metal cofactors. Besides the similar reaction mechanisms, the SAICAR synthetase from *E. coli* shares similar sequences with *E. coli* AMPSase in many regions, including the P-loop region in AMPSase (35). A sequence comparison of two proteins from *E. coli* using a computer program avairable in the GCG Sequence Analysis Package shows 24 % and 47 % for identity and similarity, respectively (data not shown). Although a relationship between aspartate and Mg^{2+} similar to that of AMPSase has not been suggested for SAICAR synthetase, the same mechanism, including Mg·Asp as an active substrate form might occur with both enzymes.

Although a regulatory role for Ca^{2+} and Mn^{2+} has been suggested for AMPSase on the basis of inhibition studies using these metal ions (36), their inhibitory effects were reexamined in this report. The inhibitory effect of Ca^{2+} seems relatively weak, and Mn^{2+} is not inhibitory (Table III). These results are inconsistent with the previous reports that

Ca^{2+} and Mn^{2+} inhibited *E. coli* (14) and mouse muscle (36) AMPSases. It is possible that the previously reported inhibition effects of AMPSase by Ca^{2+} and Mn^{2+} are overestimated due to impurities in the reagents by other metals. Nonetheless, alteration of the AMPSase activity by these metal ions at the relatively low concentration range used in these investigations, compared to the amount of Mg^{2+}, may provide information on the nature of the reaction mechanism of AMPSase. For instance, it may be possible to determine the reactivities of AMPSase in which one of two Mg^{2+} ions in the active site is replaced with another divalent metal ion.

In this study, the inhibition constant for Zn^{2+} ion (K_i) of AMPSase was estimated to be as low as 30 nM, which is slightly higher than the normal level of free Zn^{2+} ion concentration in biological systems (37). The results presented in this report imply that AMP synthesis can be inhibited by excess amounts of Zn^{2+}. This may result from abnormal nutrition. So far, no report has suggested Zn^{2+} inhibition of the formation of GMP from IMP, and the reaction mechanisms of the enzymes involved in GMP synthesis from IMP are therefore presumed not to be sensitive to this metal ion. It is conceivable that excess amounts of Zn^{2+} ion may induce inhibition of AMPSase, resulting in a decrease in the formation of only AMP in *de novo* purine synthesis. This may bring about an imbalance between GMP and AMP production from IMP. This effect is presumably associated with decreased replication fidelity (38, 39). Indeed, excess amount of Zn^{2+} ion has been reported to induce mutations in animal cells in culture (40) and chromosomal abnormalities in animals and plants (41). It is tempting to consider that Zn^{2+} ion may be involved in regulation of *de novo* GMP and AMP synthesis by inhibition of AMPSase.

In this study, we found that two divalent metal ions are identified as catalytically important in *E. coli* AMPSase. The first one may be coordinated with β- and γ-phosphoryl groups of GTP to play the role of an electron sink, whereas the second metal ion may be

complexed with aspartate in the active site. The complex formation between Mg^{2+} and aspartate in the active site is suggested for the role of the second metal ion which may be important in activation of the protonated amino group of aspartate and enhancement of the enzyme's binding affinity and specificity for aspartate. This type of activation of aspartate may also pertain to SAICAR synthetase, which has a similar mechanism and sequence to those of AMPSase. The heavy metal ion inhibition of AMPSase, which has been recognized for some time, can be explained on the formation of tight inactive complexes between the metal ions and aspartate in the active site of AMPSase. Zn^{2+} ion, which can cause strong inhibition of AMPSase, might be related to regulation of purine nucleotide synthesis and zinc cytotoxicity by excess amount of Zn^{2+} ion.

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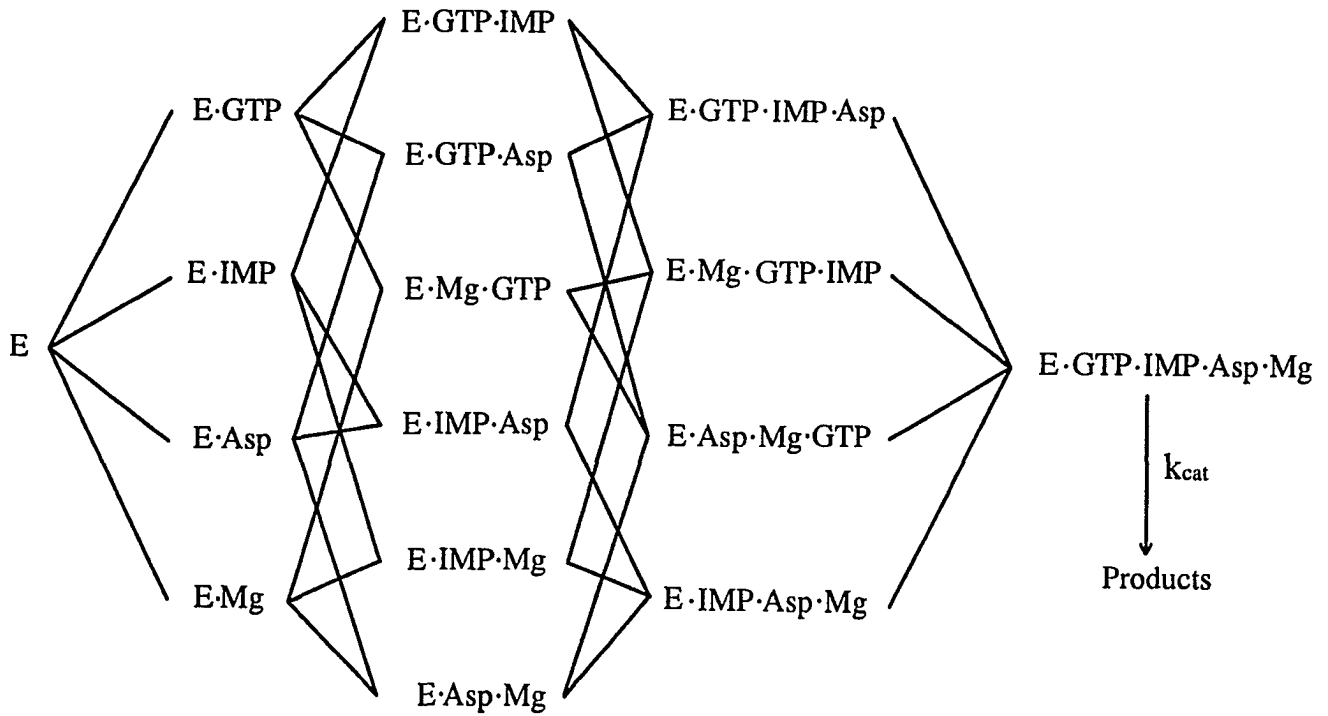

Scheme I. Kinetic Model of Adenylosuccinate Synthetase

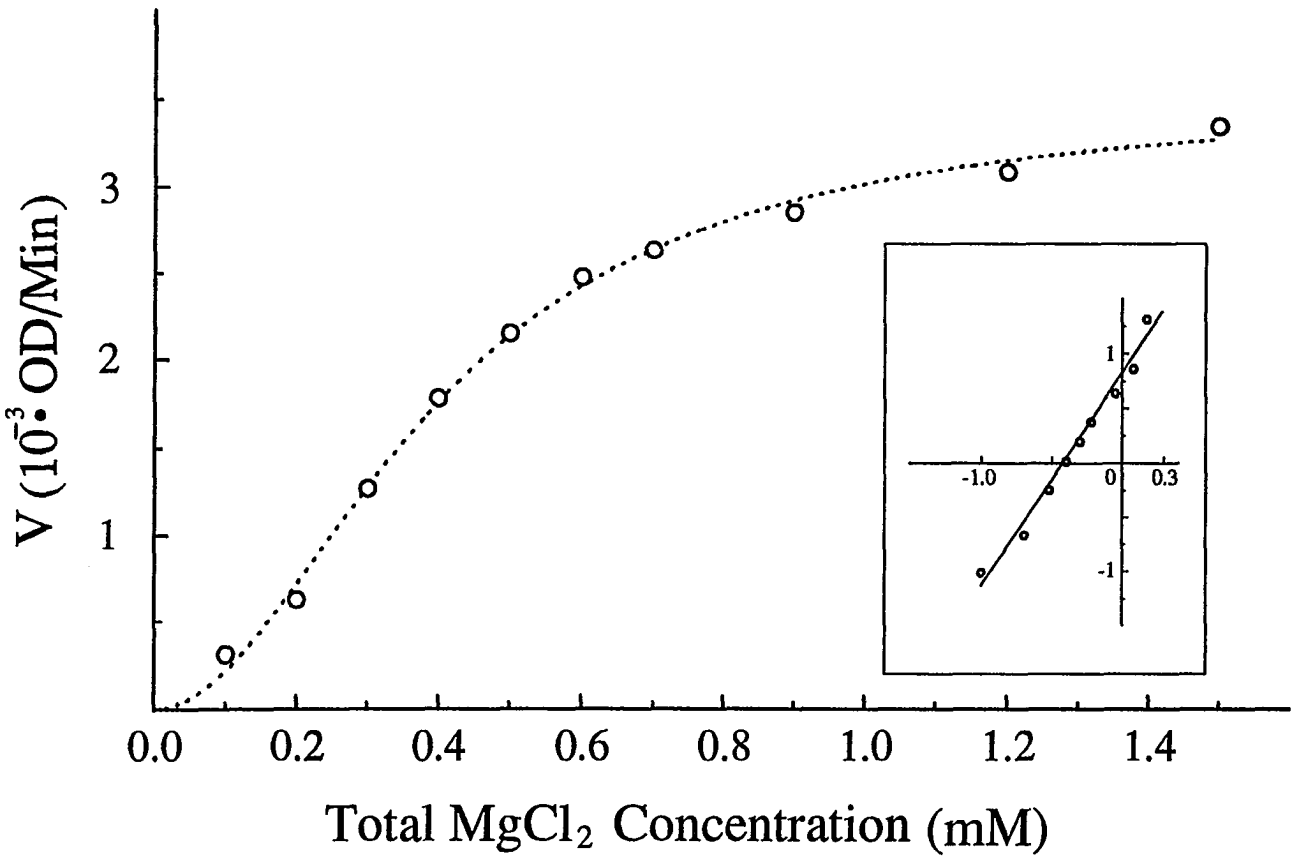

Fig. 1. The enzyme assay solution contained 40 mM Hepes (pH 7.7), 0.2 mM GTP, 0.3 mM IMP, 0.7 mM aspartate, and 1.0 μg/ml of AMPSase. The dotted line was based on a nonlinear curve fitting of the initial velocities (O) by using the Hill equation. The inset is a plot using a linear form of the Hill equation: The ordinate and the abscissa are $\log\{(V/V_{max})/(1 - V/V_{max})\}$ and $\log\{[MgCl_2]\ /(mM)\}$, respectively.

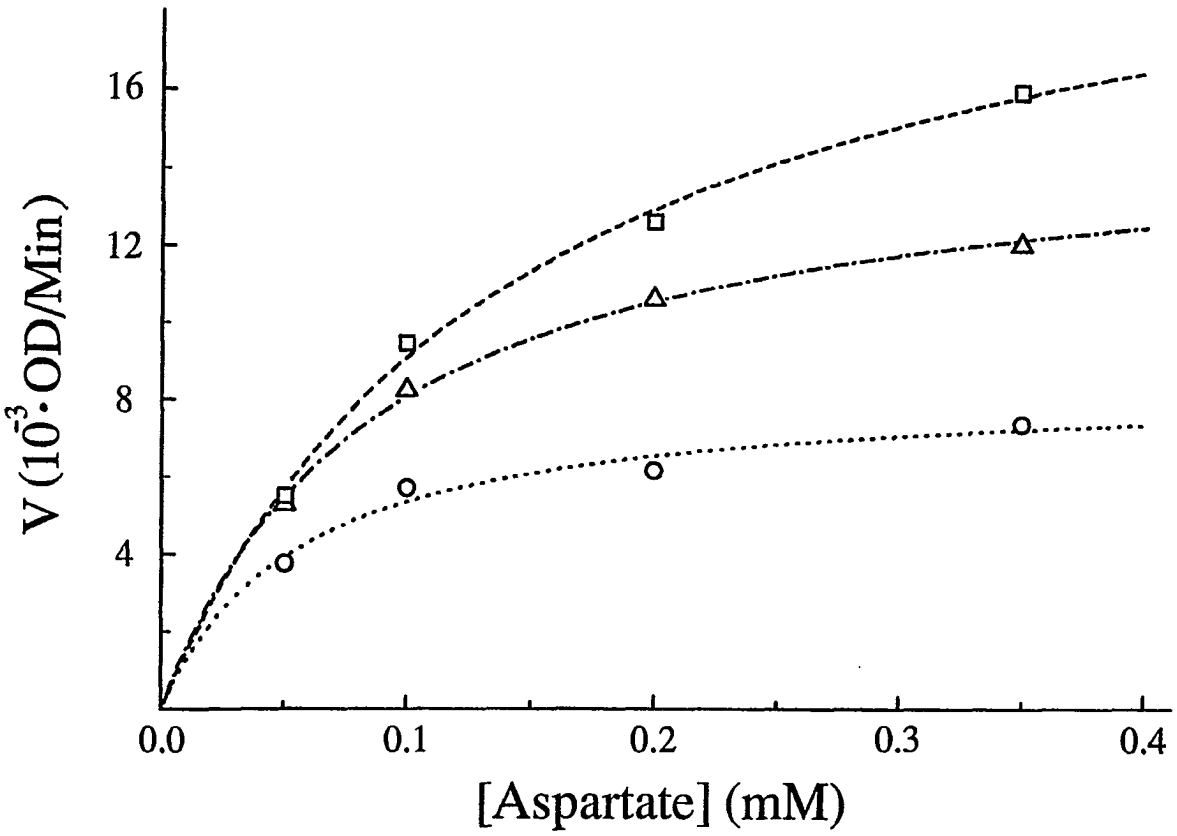

Fig. 2. The enzyme assay solution contained 40 mM Hepes (pH 7.7), 33 μM GTP, 150 μM IMP, and 2.0 μg/ml of AMPSase. The concentrations of $MgCl_2$ was fixed at the following levels: 0.30 mM, 0.60 mM, and 1.2 mM $MgCl_2$ were used for the dotted line, the mixed line, and the dashed line, respectively. The lines were drawn by fitting to the Michaelis-Menten equation.

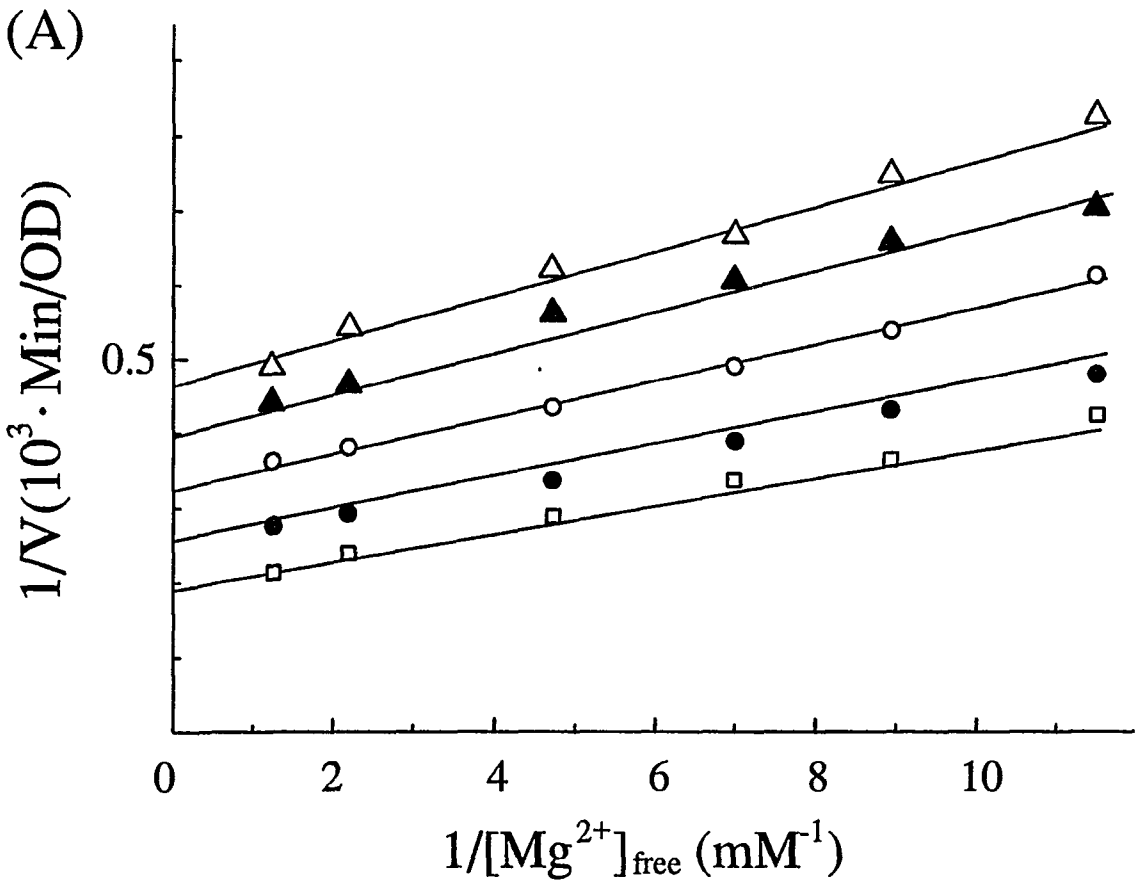

Fig. 3. Plots of the reciprocal initial velocity *versus*. $1/[Mg^{2+}]_{free}$ (A) and 1/[aspartate] (B), respectively. The lines represent theoretical fits to expeirmental data using Equation 1 and the MINITAB program (21). The activities are measured at 111 (△), 143 (▲), 200 (○), 333 (●) and 1000 (□) μM of asaprtate (A) and at 87 (△), 112 (▲), 143 (○), 212 (●), 456 (□) and 803 (■) μM of free Mg^{2+}, respectively (B). The concentrations of GTP, IMP and enzyme were 0.2 mM, 0.3 mM and 1.0 μg/ml, respectively. Other experimantal details are desribed under "Experimental Procedures".

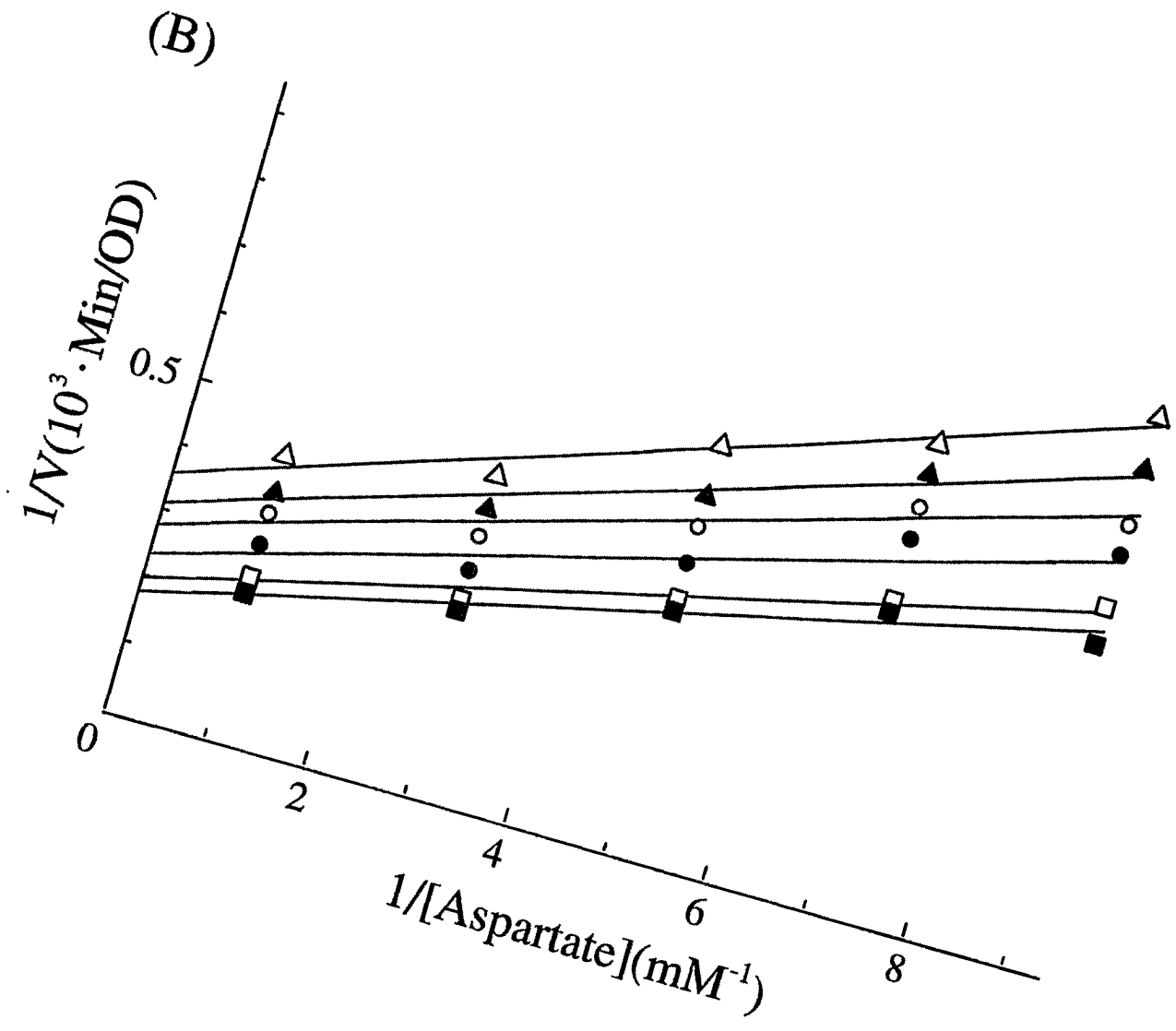

Fig 3. (Continued)

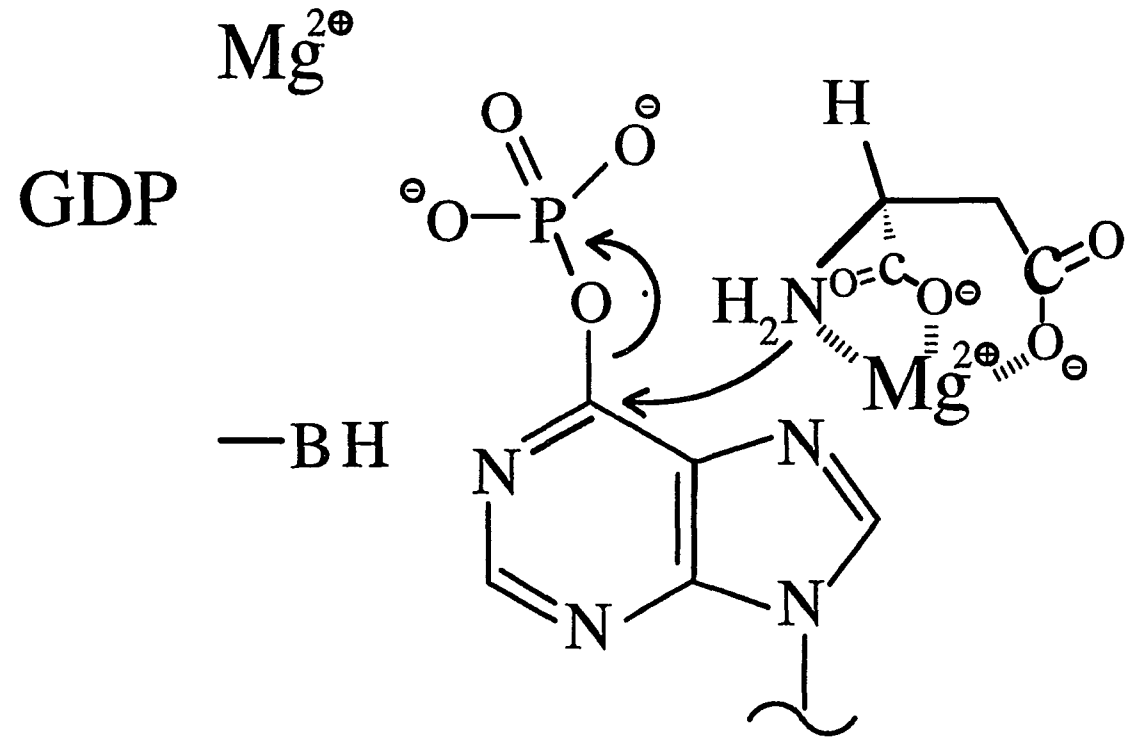

Fig. 4. A proposed model for the interactions between aspartate and Mg^{2+} in the active site of adenylosuccinate synthetase.

BH represents a protonated base (possibly, Asp^{13}). The arrows indicate movement of electron pairs during the nucleophilic substitution by the amino group of aspartate.

Table I Apparent K_m and V_{max} values for Mg^{2+} at variable concentrations of aspartate[a]

	0.1 mM Asp	0.3 mM Asp	0.5 mM Asp
$V_{max,app}$ (10^{-3} × OD/min)	1.38 ± 0.06	3.10 ± 0.08	3.59 ± 0.114
$K_{m,app}$ (μM)	119 ± 17	192 ± 15	225 ± 21

[a] The enzyme assay solution contained 40 mM Hepes (pH 7.7), 0.2 mM GTP, 0.3 mM IMP, and 1.0 μg/ml of AMPSase. The absorbance change at 290 nm was measured at 22 °C. The concentration of Mg^{2+} was varied using $MgCl_2$, and free Mg^{2+} concentration was calculated by using an iteration program described under "Experimental Procedures".

Table II Kinetic parameters of *E. coli* adenylosuccinate synthetase[a]

k_{cat}	$K_{d,Asp}$	$K_{d,Mg}$	$K_{m,Asp}$	$K_{m,Mg}$
1.47 ± 0.07/sec	79.2 ± 19.1 μM	40.0 ± 9.4 μM	225 ± 16 μM	114 ± 26 μM

[a] The kinetic parameters were deduced from a statistical analysis of the data shown in Fig. 3(A) and (B) by using a MINITAB computer program (21).

Table III Metal ion effects on the kinetic parameters of the wild-type AMPSase[a]

	$k_{cat,av}$ (/sec)	$K_{m,GTP}$ (μM)	$K_{m,IMP}$ (μM)	$K_{m,Asp}$ (μM)
$MgCl_2$	1.26 ± 0.15	16.0 ± 1.1	18.3 ± 0.7	225 ± 16
$CaCl_2$	0.235 ± 0.091	2.72 ± 0.56	9.87 ± 1.61	33.7 ± 3.4
$MnCl_2$	0.930 ± 0.047	6.54 ± 0.83	13.9 ± 6.0	46.8 ± 8.3

[a] The enzyme assay solution contained 40 mM Hepes (pH 7.7), 1.0 μg/ml (for the kinetics using Mg^{2+} and Mn^{2+}) or 6.4 μg/ml (for the kinetics using Ca^{2+}) of AMPSase and 2.0 mM of the corresponding metal ions as their chloride salts. When the concentration of GTP, IMP or aspartate was unvaried, it was held at 0.20 mM, 0.30 mM or 2.0 mM, respectively. Data fitting was performed using a nonlinear regression program (ENZFITTER) (22) based upon the Michaelis-Menten model.

Table IV Kinetic parameters of adenylosuccinate synthetse in the presence of $ZnCl_2$[a]

	$V_{max,app}$ (10^{-3}·OD/min)	$K_{m,app}$ (mM)
Variable Asp, 0.0 μM $ZnCl_2$	12.1 ± 0.5	0.093 ±.0018
Variable Asp, 0.5 μM $ZnCl_2$,	8.76 ± 0.62	0.328 ± 0.082
Variable Mg^{2+}, 0.0 μM $ZnCl_2$	7.92 ± 0.24	0.117 ± 0.025
Variable Mg^{2+}, 0.5 μM $ZnCl_2$[b]	5.35 ± 0.32	0.515 ± 0.101
Variable IMP, 0.0 μM $ZnCl_2$	9.42 ± 0.65	0.0604 ± 0.0104
Variable IMP, 0.5 μM $ZnCl_2$	4.53 ± 0.21	0.0423 ± 0.0053
Variable GTP, 0.0 μM $ZnCl_2$	9.28 ± 0.28	0.0121 ± 0.0014
Variable GTP, 0.5 μM $ZnCl_2$	5.92 ± 1.49	0.0109 ± 0.0015

[a] The enzyme assay solution contained 40 mM Hepes (pH 7.7) and 2.0 μg/ml of AMPSase. The absorbance change was measured at 280 nm. When the concentration of GTP, IMP, aspartate or Mg^{2+} was unvaried, it was held at 0.033 mM, 0.15 mM, 0.50 mM or 2.0 mM, respectively.

[b] Free Mg^{2+} and Zn^{2+} concentrations were calculated by using an iteration program described under "Materials and Methods" to deduce the apparent K_i value for Zn^{2+} which is defined as $[Zn^{2+}] \times K_{m,0}/(K_{m,0.5} - K_{m,0})$ and where $K_{m,0}$ and $K_{m,0.5}$ represent the K_m values in the absence and presence of 0.5 μM of $ZnCl_2$. Under these conditions, the concentration of free Zn^{2+} was estimated to be as low as 0.10μM.

GENERAL CONCLUSION

In this dissertation, it was shown that the GTP-binding consensus sequences found in AMPSase are important in catalysis and substrate binding. Particularly, Asp^{333} is a key determinant in the GTP specificity of this enzyme. Also, for the first time, it has been established that a second metal ion, in addition to the metal ion coordinated with the phosphate group of GTP, is involved in the reaction mechanism of AMPSase, presumably in a form of a complex with Asp.

Considering the roles of the GTP-binding consensus sequences found in AMPSase, the residues belonging to the P-loop region are obviously associated with catalysis as demonstrated in Chapter I of this dissertation and a mutational study in this region by our laboratory (35). Interestingly, the CD spectrum of the G15V mutant was altered by the presence of either GTP or IMP. It is believed that a conformational change which is reflected by changes in the CD spectrum is associated with the low activity of the mutant, whereas the wild-type did not show any major changes in CD spectra under similar conditions. This result indicates that the P-loop region is also involved in conformational changes of the enzyme by GTP and IMP. The possibility of participation of the P-loop in the conformational changes induced by substrates can be further supported by analysis of other mutants in the P-loop, which are currently in progress (unpublished results). For instance, the mutation of the Asp residue at the 13 position in AMPSase to Ala resulted in no detectable activity using UV-spectrophotometer, but the inactive enzyme retains the ability to bind GTP with even higher affinity than does the wild-type enzyme. The fluorescence intensity of the same mutant was only 20 % of the wild-type protein under the same conditions although it's global structure was not changed during mutation as shown in comparison of the CD specra for the D13A mutant and wild-type AMPSase. These results

indicate that besides it's catalytic role, the β-carboxyl group of the Asp residue seems to stabilize other structural elements in the protein. One can speculate that a strong correlation exists between the higher affinity for GTP and the destabilization of some structural elements found in this mutant. In order to explain the correlation, it may be adequate to suggest that the β-carboxyl group of the Asp residue at position 13 may couple GTP-binding with a conformational change that is essential in catalysis. Mutation of Asp^{13} may block utilization of GTP-binding energy for catalysis, resulting in the higher binding constant observed for GTP in the D13A mutant protein. Taken together, these results make it possible to suggest that the P-loop region is a key element transforming substrate binding energy into catalytic energy by orchestrating the conformational changes of the protein induced by substrates. However, further investigations will be necessary to confirm this suggestion.

Another consensus sequence for GTP binding present in AMPSase exists as the $T^{330}KXD^{333}$ box. The Asp residue is completely conserved among the known sequences of the proteins. As shown in Part I of the dissertation, the Lys residue in this region is apparently responsible for the GTP binding affinity of this protein through hydrophobic interactions between its linear side chain and the guanine base of GTP, although it appears not to be essential for catalysis despite it's conservation among the AMPSase sequences. This result is similar to that found from a mutational analysis of the corresponding residue in $p21^{ras}$ (43). However, an unusual feature found in the mutants at the Lys residue in *E. coli* AMPSase is that both the K331L and K331R mutants have extremely high K_m values for Asp as well as for GTP. Thus, the terminal positive charge in the side chain of the Lys residue seems not to be important. Although the reason for these high K_m values for Asp of the mutants is not apparent, it may be related to non-linearity of the residues in the mutant enzymes. The environment of the Lys residue in the wild-type enzyme may be compact so

that only linear side chains are allowed at this position. In fact, Ser and Met appear at this position in the sequences of AMPSase from some sources (30, B. Labedan, personal communication, respectively).

In Chapter II of the dissertation, the role of Asp in the consensus sequence $TKXD^{333}$ in AMPSase was discussed. The Asp residue turned out to be a key determinant of the exquisite specificity of the enzyme for GTP using combinations of the mutants at this position and GTP-analogs. Considering that similar results were reported for other GTP binding proteins such as $p21^{ras}$ (40) and elongation factor Tu (41), and the structural similarity among these proteins (37, 44), it is plausible that the Asp residues in the conserved N(T)KXD boxes among the GTP-binding proteins, including AMPSase, are extremely similar in their functions and structures.

The Asp residue at the position 333 in AMPSase is also involved in catalysis although it is not clear how a residue 20 Å away from the catalytic site participates in catalysis. The catalytic role of this residue became more certain by a comparison of kinetic parameters of the mutant and wild-type enzymes using GTP, ITP and XTP. When GTP is selected as a substrate, the Michaelis-Menten complexes of the mutants are energetically more activated compared to that of the wild-type enzyme as demonstrated by their increased K_m values. However, the k_{cat}/K_m values of the mutant proteins are significantly decreased by the mutations, which indicates that the transition state of the wild-type enzyme with GTP is more stabilized than those of the mutant proteins with GTP. In the case of ITP, it is obvious that the stabilization of the Michaelis-Menten complexes of the mutants results in lower activities without significant changes in the energy levels of the transition state. This result indicates that the type of the interactions of the residues at position 333 with the base of ITP may be important, not for transition state stabilization, but only for ground state stabilization (the stabilization of the Michaelis-Menten

complexes). Using XTP, a similar analysis indicates that there is an interaction between the Asn residue at the position 333 with the xanthine base of XTP and that this interaction contributes mainly to stabilization of the transition state. Likewise, interaction of the residue at the position 333 with the base of the nucleotide has a great influence on the stabilization of transition state as well as the ground state of AMPSase.

In Chapter III of this dissertation, it was demonstrated that AMPSase needs two metal ions in the enzyme active site. One metal ion is assumed to coordinate with the β- and γ-phosphoryl groups of GTP whereas the other is associated with Asp. The function of the latter metal ion is suggested to enhance nucleophilicity of the amino group of Asp, which is presumed to be essential for the AMPSase reaction. It has been known for a long time that in basic solution, Asp and Mg^{2+} ion forms a Asp·Mg complex. An X-ray diffrection study of the Asp·Mg complex (45) reveals that the amino group of Asp is coordinated with Mg^{2+} ion in it's deprotonated form. Thus, the pK_a value of the amino group will be lowered by complex formation. If one assumes that the active site of AMPSase allows Asp to form a similar complex with Mg^{2+} ion, the reported literature values for the formation constant of Asp·Mg (46) suggests that the pK_a value of the amino group in the enzyme active site will be around 7. This value is low enough to allow a significant fraction of the population of amino groups of Asp to be deprotonated at pH 7.7. This suggestion nicely explains the requirement of a base to remove the proton on the α-amino group and the β-carboxyl group of Asp (18) and the preferred *cis*-conformation of the two carboxyl groups of Asp (47). Recently, a complex of Asp with Mg^{2+} ion was found in the active site of *E. coli* AMPSase as revealed by an X-ray diffraction study (Richard B. Honzatko, personal communication). Although the complex looks slightly different from the Asp·Mg complex in the absence of the enzyme, this study confirms the presence of a complex between Asp and Mg^{2+} in the active site of AMPSase. Besides, it is

also demonstrated in this dissertation that Zn^{2+} ion inhibition of AMPSase is caused by Zn^{2+} occupation at the second metal ion binding site (an inactive comlex with Asp).

The syntheses of AMP and GMP from IMP require four different proteins where each branch requires two enzymes. The apparent inhibition constant of Zn^{2+} ion for AMPSase is extremely low (30 nM). Also, the other enzyme involved in the AMP synthesis, adenylosuccinate lyase, can be inhibited by the presence of trace heavy metals *in vitro* (48). Although the inhibition constant of Zn^{2+} ion in adenylosuccinate lyase reaction has not been established quantitatively, the fact that this metal ion can inhibit two consecutive enzymes in a metabolic pathways may make Zn^{2+} ion an even more potent inhibitor in the synthesis of AMP from IMP than was previously thought. The inhibition by Zn^{2+} ion of two enzymes in the synthesis of AMP from IMP may regulate the balance of the GMP and AMP ratio *in vivo*. Obviously, more detailed information will be necessary before this hypothesis can be verified.

In summary, it is shown that Lys^{331} interacts with GTP through hydrophobic interactions between its linear side chain and the aromatic ring of the guanine base of GTP and that the phosphate-binding region of AMPSase is involved in a conformational change induced by GTP and IMP binding that is required for catalysis. The aspartate residue at position 333 of *E. coli* AMPSase is identified as a key determinant in the recognition of nucleoside triphosphates, and by modest changes in the side chain of the residue 333, the wild-type, GTP-hydrolyzing enzyme is transformed into an even more proficient XTP-hydrolyzing enzyme. A stoichiometric study of the metal ions in AMPSase using kinetics revealed, for the first time, that the second metal ion is involved in the reaction mechanism of AMPSase through complex formation with Asp. However, in order to describe the more detailed reaction mechanism of AMPSase, further investigation will be necessary.

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