Replacement of Asp<sup>333</sup> with Asn by Site-directed Mutagenesis Changes the Substrate Specificity of <i>Escherichia coli</i> Adenylosuccinate Synthetase from Guanosine 5'-Triphosphate to Xanthosine 5'-Triphosphate*  

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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<sup>333</sup> of the synthetase from <i>Escherichia coli</i> is changed to asparagine, glutamate, and glutamine by site-directed mutagenesis. The mutants D333N, D333E, and D333Q generally show decreased <i>k</i><sub>cat</sub> values and increased <i>K</i><sub>m</sub> values for GTP. The decreased values of <i>k</i><sub>cat</sub> exhibited by the mutants indicate that the interactions between Asp<sup>333</sup> and the guanine are relayed by some mechanism to the catalytic residues around the <i>y</i>-phosphate of GTP, and that the energy provided by the interconversion between Asp<sup>333</sup> and the guanine moiety of GTP is utilized for rearrangement of the catalytic residues. The three mutants each have higher affinity for xanthosine 5'-triphosphate (XTP) 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<sup>333</sup> forms hydrogen bonds with the N-1 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<sup>333</sup> and N-1 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 glutamate at residue 333 favors pyridimine nucleotides over the purine nucleotides, GTP, XTP, and ITP. These results demonstrate that Asp<sup>333</sup> in the (N/T)KXD consensus sequence of adenylosuccinate synthetase from <i>E. coli</i> is a determinant for GTP-specificity.

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 variants of (N/T)KXD consensus sequence interacts with the C-2 exocyclic amino group and a hydrogen atom of the N-1 endo-nitrogen of the guanine base of GTP. A mutation of aspartate to asparagine at the corresponding residue in p21<sup>0</sup> (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 <i>E. coli</i> AMPSe 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**—<i>E. coli</i> strain XLI-Blue came from Stragen, a site-directed mutagenesis kit from Amersham Corp., and restriction enzymes from Promega. GTP, UTP, ITP, XTP, and Cibacron blue 3GA were obtained from Sigma, and pur <i>A</i> 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 procedure previously described (17). The mutated plasmids were transformed into an <i>E. coli</i> pur <i>A</i> strain (H1238), which does not produce AMPSe, in order to prevent mutant protein contamination by wild-type enzyme.

Preparation and Kinetics of Wild-type and Mutant AMPSe—The mutant forms of AMPSe were purified by modification of the procedure described previously (22). Phenyl-Sepharose, affinity chromatography, using a Cibacron 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 a molar extinction coefficient for wild-type AMPSase at 280 nm ($e_{280} = 67.85 \text{mM}^{-1} \text{cm}^{-1}$), where the concentration refers to monomers. AMPSase activity was determined as described earlier (24), except that the absorbance change at 289 nm was monitored. 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 pg/ml enzyme was used depending on the activity of each mutant.

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. The procedure used in this study was similar to that previously described (17). The protein concentration was 0.10-0.15 mg/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 AMPSase 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 structure (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 Cibacron 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 96% 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 AMPSase 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 $K_v$ 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, $\Delta_{272}$ at 254 nm, 272 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 h after the reaction was initiated. The absorbance change indicates 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 96%. 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 10 mM. As the only difference between ITP and GTP is the exocyclic amino group at C-2, the interaction of this amino group with AMPSase may be critical to substrate recognition. $K_v$ values for ATP were 193 $\mu$m for D333N, 140 $\mu$m for D333E, and 334 $\mu$m for D333Q. These results show that Asp$^{333}$ of AMPSase is apparently involved in GTP binding, providing 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/s, 0.0189/s, and 0.0179/s, respectively, using GTP as a substrate. Given that the x-ray results place the
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**Table III**

**Comparison of the wild-type and Asp<sup>333</sup> mutant AMPSases**

A typical enzyme assay solution contained 450 µM IMP, 5 mM Asp, 40 mM Hepes (pH 7.7), 5 mM MgCl<sub>2</sub>, and appropriate amount of AMPSases.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;m,GTP&lt;/sub&gt; (mM)</th>
<th>K&lt;sub&gt;m,UTP&lt;/sub&gt; (mM)</th>
<th>k&lt;sub&gt;m&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;m,ITP&lt;/sub&gt; (mM)</th>
<th>K&lt;sub&gt;m,XTP&lt;/sub&gt; (mM)</th>
<th>k&lt;sub&gt;m&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.733 ± 0.096</td>
<td>22.2 ± 5.4</td>
<td>0.011 ± 0.009</td>
<td>2.08 ± 0.40</td>
<td>0.628 ± 0.17</td>
<td>17.2 ± 5.4</td>
<td>0.0258 ± 0.0016</td>
<td>388 ± 63</td>
</tr>
<tr>
<td>D333N</td>
<td>0.111 ± 0.003</td>
<td>193 ± 10.9</td>
<td>0.0210 ± 0.0015</td>
<td>1.27 ± 0.18</td>
<td>0.0622 ± 0.0067</td>
<td>3.29 ± 0.64</td>
<td>1.14 ± 0.07</td>
<td>33.1 ± 5.2</td>
</tr>
<tr>
<td>D333E</td>
<td>0.0189 ± 0.0029</td>
<td>140 ± 44</td>
<td>0.011 ± 0.003</td>
<td>4.82 ± 2.59</td>
<td>0.0743 ± 0.0091</td>
<td>2.87 ± 0.69</td>
<td>0.0523 ± 0.0017</td>
<td>28.5 ± 3.5</td>
</tr>
<tr>
<td>D333Q</td>
<td>0.0179 ± 0.0015</td>
<td>334 ± 73</td>
<td>0.0978 ± 0.0192</td>
<td>3.41 ± 0.97</td>
<td>0.0439 ± 0.0031</td>
<td>1.07 ± 0.23</td>
<td>0.0599 ± 0.0044</td>
<td>54.7 ± 8.3</td>
</tr>
</tbody>
</table>

<sup>*</sup> MgCl<sub>2</sub>, 15 mM, was used.

**Table IV**

**Comparison of specificity constants of the AMPSases for GTP and XTP**

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Specificity constant for GTP (k&lt;sub&gt;m&lt;/sub&gt;GTP/K&lt;sub&gt;m&lt;/sub&gt;)</th>
<th>Specificity constant for XTP (k&lt;sub&gt;m&lt;/sub&gt;XTP/K&lt;sub&gt;m&lt;/sub&gt;)</th>
<th>Relative substrate preference (RSP) (XTP/GTP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>3.30 ± 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>2.09 ± 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D333N</td>
<td>5.75 ± 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>59.8</td>
<td>(2.86 ± 10&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D333E</td>
<td>1.35 ± 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>13.6</td>
<td>(6.51 ± 10&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D333Q</td>
<td>5.36 ± 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>20.5</td>
<td>(9.82 ± 10&lt;sup&gt;-2&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative values; RSP (D333N)/RSP (wild-type).  

![UV-visible difference spectra of the AMPSase reaction of the D333N mutant with XTP at different times.](image)

Fig. 2. UV-visible 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<sub>2</sub>, 0.05 mM XTP, 2 µg of D333N mutant, and 10 µM of Hepes buffer (pH 7.7). Arrows indicate absorbance changes with increasing time.

Replacement of Asp<sup>333</sup> 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 XTP and ATP as substrates relative to the wild-type enzyme. The 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 (NTK)XKD 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 \( k_m/K_m \) 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 Asp333 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 interaction of GTPD 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 \( \gamma \)-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 GDP. 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 AMP-Sases with nucleotides including XTP may provide information regarding these conformational changes in the synthetase.

Previously, our laboratory has reported that two mutants at Lys331 (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 Lys331, the Asp333 mutants have greatly reduced activity as well as reduced affinity for GTP. Apparently, the interaction energy of Lys331 with GTP is used only for binding, but that of Asp333 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 GDP 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 Asp333. 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 \( K_m \) as a criterion. In addition, we report that Asp333 of (NTK)XKD in E. coli AMPSase contributes directly to ATP-binding as well as to catalysis through long-range interactions. By mutating this residue to Asn, Glu, and Gin, 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 Lys331 mutants previously reported by our laboratory (17), a portion of the binding energy derived from the interaction of Asp333 with guanine of GTP may be used in reorganizing catalytic residues.

Note Added in Proof—Since submission of this research for publication, Weiland and Parmeggiani (30) 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 between Asn189 and XTP to a report by Hwang and Miller (20); however, these investigators did not suggest the formation of a 6-membered hydrogen bonding network between Asn189 and XTP in their study.

REFERENCES