**Relationship of Conserved Residues in the IMP Binding Site to Substrate Recognition and Catalysis in *Escherichia coli* Adenylosuccinate Synthetase***

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Gln\(^{34}\), Gln\(^{224}\), Leu\(^{228}\), and Ser\(^{240}\) are conserved residues in the vicinity of bound IMP in the crystal structure of *Escherichia coli* adenylosuccinate synthetase. Directed mutations were carried out, and wild-type and mutant enzymes were purified to homogeneity. Circular dichroism spectroscopy indicated no difference in secondary structure between the mutants and the wild-type enzyme in the absence of substrates. Mutants L228A and S240A exhibited modest changes in their initial rate kinetics relative to the wild-type enzyme, suggesting that neither Leu\(^{228}\) nor Ser\(^{240}\) play essential roles in substrate binding or catalysis. The mutants Q224M and Q224E exhibited no significant change in \(K_{m}\) for ASP and \(K_{m}\) for IMP and modest changes in \(K_{cat}\) relative to the wild-type enzyme. However, \(k_{cat}\) decreased 13-fold for the Q224M mutant and 10-fold for the Q224E mutant relative to the wild-type enzyme. Furthermore, the Q224E mutant showed an optimum pH at 6.2, which is 1.5 pH units lower than that of the wild-type enzyme. Tryptophan emission fluorescence spectra of Q224M, Q224E, and wild-type enzymes under denaturing conditions indicate comparable stabilities. Mutant Q34E exhibits a 60-fold decrease in \(k_{cat}\), compared with that of the wild-type enzyme, which is attributed to the disruption of the Gln\(^{34}\) to Gln\(^{224}\) hydrogen bond observed in crystal structures. Presented here is a mechanism for the synthetase, whereby Gln\(^{224}\) works in concert with Asp\(^{23}\) to stabilize the 6-oxanyon of IMP.

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Adenylosuccinate synthetase (IMP:L-aspartate ligase (GTP-forming), EC 6.3.4.4, AMPase)\(^{1}\) catalyzes the first committed step in de novo AMP synthesis, using GTP as the energy source to couple IMP and aspartate (1). The synthetase is the target of a natural herbicide (2–5), and the enzyme is a potential target for the formation of 6-phosphoryl-IMP (19, 23, 24) as an intermediate in the catalytic reaction. O-6 of IMP putatively makes a nucleophilic attack on the \(\gamma\)-phosphorus atom of GTP, whereupon the amino group of L-aspartate (ASP) displaces phosphate from C-6 of the intermediate. The direct observation of 6-thiophosphoryl-IMP in the active site of the synthetase (20) provides additional support for the proposed mechanism, which, on the basis of initial rate kinetics, is rapid equilibrium random ter ter (25). Isotope exchange studies with the rat muscle enzyme imply a preferred pathway for substrate addition, with aspartate adding to the enzyme after the binding of nucleotides (24). Competitive inhibitors for one of the substrates inhibit noncompetitively with respect to the other two substrates, suggesting that substrate binding sites do not overlap (Ref. 1 and references therein). Furthermore, adenylosuccinate (AMPs) is a competitive inhibitor of IMP and a noncompetitive inhibitor of GTP and ASP, a result consistent with overlapping AMPs and IMP binding sites (25).

Residues located in the vicinity of C-6 of IMP should be important in catalysis and/or substrate binding. Directed mutation, kinetics, and molecular modeling (19, 26) revealed the specificity of Arg\(^{205}\) to the recognition of ASP and the stabilization of the transition state. The putative binding of Arg\(^{205}\) to the \(\alpha\)-carboxylate of ASP may bring the amino group of ASP into close contact with C-6 of IMP (19). However, Arg\(^{205}\) is probably not a significant factor in the formation of 6-phosphoryl-IMP. Positions corresponding to 224 are occupied either by Gln or Asn in 12 of the 13 known sequences of AMPsase. The side chain of Gln\(^{224}\) hydrogen bonds to N-7 and O-6 of IMP (19). On the basis of results presented below, Gln\(^{224}\) is essential for catalysis. Furthermore, the mutation of Gln\(^{224}\) to glutamate causes a 60-fold decline in \(k_{cat}\), attributed to the disruption of a hydroxide bond between Gln\(^{34}\) and Gln\(^{224}\), that was observed in ligated crystal structures (19). In contrast, alanine mutations of Leu\(^{228}\) and Ser\(^{240}\), which are conserved residues near bound IMP, had little effect on the kinetics of the synthetase.

**EXPERIMENTAL PROCEDURES**

**Materials**—GTP, IMP, L-aspartate, adenylosuccinate, phenylmethylsulfonyl fluoride, and bovine serum albumin were obtained from Sigma. A site-directed mutagenesis kit was obtained from Amersham Corp. Restriction enzymes were obtained from Promega. *E. coli* strain XL-1 blue was obtained from Stratagene. An *E. coli* purA strain H1238 (thr-25, tonA40, argF58, purA34, argB61) and an *E. coli* purB strain H680 (fhuA2, lacY1, thi-76, gluV44, asf1, gal-6, l-lys, purB51, trpC54, his-6, tyrA2, rplL125strR1, nifA1, xylA7, mtlA2, thi-1) were obtained from Dr. B. Bachman, Genetic Center, Yale University. 0.45-μm polyvinylidene difluoride membranes were obtained from Millipore Corp. Other reagents and chemicals were obtained from Sigma.

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\(^{1}\) The abbreviations used are: AMPsase, adenylosuccinate synthetase; ASP, l-aspartate; AMPs, adenylosuccinate; Mes, 4-morpholineethanesulfonic acid.
Site-directed Mutagenesis—Recombinant DNA manipulation employed standard procedures (27). The plasmid containing a 1.8-kilobase pair BamHI-HindIII fragment from FMS204, ligated into PUC118, was used in mutagenesis. The mutagenesis primers were as follows: 5'-gagctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgct
proposals regarding the catalytic mechanism of the synthetase and the role in that mechanism played by the side chain at position 224. Mes and Hepes were chosen as buffers for the pH range 5.5–8.5, where the wild-type enzyme is active. The assay solution contained 300 μM GTP, 5 mM MgCl2, 5 mM ASP, and 20 mM Mes/Hepes buffer at different pH values. IMP concentrations varied from 25 to 500 μM, 300 μg/ml Q224E mutant or 1 μg/ml wild-type AMPase with excess AMP lyase were used in the assays. Longer assay times (5–10 min) compensated for the lower activity of the Q224E mutant enzyme.

RESULTS

Sequence Conservation of Gln34, Gln224, Leu228, and Ser240—Sequences of AMPase from different sources were aligned using the PILEUP option of the GCG sequence analysis package. Positions equivalent to 224 are occupied by asparagine in four sequences, glutamine in eight sequences, and arginine in the AMPase from Brucella abortus, suggesting a significant role for the amide side chain at this position (Table I). Indeed, Gln224 hydrogen bonds to the 6-oxo and N-7 of IMP in a crystal structure of the ligated synthetase (19). Position 34 is occupied by glutamine in 10 of 13 sequences. In ligated crystal structures of the E. coli enzyme, NE2 of Gln34 hydrogen bonds to OE1 of Gln224, properly orienting the amide side chain of Gln224 not only in the recognition of IMP but also in a critical catalytic function. The catalytic role of Gln 224 had not been identified as a mutant of AMPase by Western blot and complementation experiments using H680 and H1238 as the recipient strains. The wild-type, Q224M, and Q224E enzymes were recognized by the antibody for AMPase, whereas AMPases lyase was not (data not shown). In M9 minimal medium, the Q224M, and Q224E mutant plasmids complemented adenine auxotrophy of purA− in H1238 but not purB− in H680.

Secondary Structure Analysis—The CD spectra of the mutant and wild-type enzymes were superimposable (data not shown) from 200 to 260 nm. These observations indicated the absence of global conformational changes or the disruption of the secondary structure.

Kinetic Characterization of Wild-type and Mutant AMP-Sases—The kinetic parameters for wild-type and mutant AMP-Sases were determined in pH 7.7 Hepes buffer (Table II). No significant change in the Km values for GTP or ASP was observed for Q224E or Q224M enzymes relative to the wild-type enzyme. The Q224M mutant displayed a 5-fold increase in Km for all three substrates, exhibited a decrease in kcat of 4 orders of magnitude relative to wild-type AMPase. Specificity constants (kcat/Km) for the Q224M mutant decreased 16-, 75-, and 14-fold for GTP, IMP, and ASP, respectively, relative to the wild-type enzyme, compared with an almost 104-fold decrease for specificity constants of the Q224E mutant for all substrates. These findings implicate Gln224 not only in the recognition of IMP but also in a critical catalytic function. The catalytic role of Gln224 had not been recognized previously on the basis of ligated crystal structures.
but it is in harmony with the suggestion of Poland et al. (19) that IMP binds to the active site as the 6-oxyanion (see below).

The substitution of Gln34 with glutamate resulted in a 60-fold decrease in \( k_{cat} \). Atom N-E2 of Gln34 hydrogen bonds with O-E1 of Gln224, which putatively orients Gln224 in its interaction with IMP. The Q34E mutant may force a 180° rotation of the amide side chain of Gln224 (see below). However, in the case of the Q34E mutant, one cannot rule out other explanations for the observed loss of activity, since the \( K_m^{\text{GTP}} \) also increased 20-fold relative to the wild-type enzyme. Gln34 is about 10 Å away from the GTP site, suggesting a conformational change over a significant distance.

Alanine substitution of Leu228 and Ser240 resulted in 3.6- and 1.4-fold decreases in \( k_{cat} \), respectively, relative to the wild-type enzyme. The side chain of Leu228 may contribute to the proper orientation of the base of IMP; however, Ser240 apparently plays no significant role in the recognition of IMP or in catalysis.

**Intrinsic Tryptophan Emission Fluorescence Measurements**—The fluorescence emission spectra of the mutants, Q34E, Q224M, and Q224E, and the wild-type enzyme were collected in the presence of different concentrations of guanidine chloride. The emission maximum was 333 nm for the enzymes to correlate enzyme activity with the state of protonation of essential catalytic residues severely inhibits the enzyme at pH 6.2.

As the \( K_m \) values for substrates are not significantly perturbed by the mutation Q224M, the decrease in \( k_{cat} \) most likely emanates from the loss of hydrogen bonds to N-7 and O-6 of IMP, the latter probably having the largest impact on catalysis (Fig. 4C). Partial restoration of activity at pH 6.2, then, is due putatively to the protonation of Gln224, which reforms the critical hydrogen bond to O-6 of IMP (Fig. 4D). The failure to restore the Q224E mutant to 100% of the wild-type enzyme activity at pH 6.2 may be a consequence of (i) tautomeric states that put the proton onto O-6 (Fig. 4E) and/or (ii) the absence of the N-7 to Gln224 hydrogen bond, an absence that may undermine the stability of the hydrogen bond between Gln224 and O-6 of IMP.

The physiological importance of Gln224 is supported further by the phenomenon of reduced cell proliferation in combination with enhanced protein expression for the mutant Q224E. Mutation of essential catalytic residues severely inhibits the growth of *E. coli* in the case of D13A and H41N mutants (35). The kinetic data imply that a disruption of the purine nucleotide pathway may impair the reproductive capacity of the organism.

**TABLE II**

| Protein | \( s^{-1} \) | \( K_m^{\text{GTP}} \) | \( K_m^{\text{IMP}} \) | \( K_m^{\text{AMP}} \) | \( k_{cat}/K_m^{\text{GTP}} \) | \( k_{cat}/K_m^{\text{IMP}} \) | \( k_{cat}/K_m^{\text{AMP}} \) |
|---------|-------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Wild type | 1.00 ± 0.05 | 52.5 ± 6.21 | 59.6 ± 4.62 | 0.35 ± 0.02 | (1.87 ± 0.23) × 10^{-2} | (1.68 ± 0.15) × 10^{-2} | 2.87 ± 0.22 |
| Q34E | (1.65 ± 0.00) × 10^{-2} | (1.09 ± 0.02) × 10^{-2} | 78.9 ± 7.71 | 0.52 ± 0.04 | (1.54 ± 0.03) × 10^{-2} | (2.41 ± 0.20) × 10^{-4} | (3.23 ± 0.30) × 10^{-2} |
| Q224M | 0.07 ± 0.00 | 60.5 ± 7.32 | 316 ± 42.1 | 0.35 ± 0.04 | (1.16 ± 0.14) × 10^{-2} | (2.22 ± 0.30) × 10^{-4} | (2.00 ± 0.12) × 10^{-1} |
| Q224E | (1.76 ± 0.13) × 10^{-4} | 68.9 ± 6.10 | 50.5 ± 5.87 | 0.32 ± 0.05 | (2.55 ± 0.29) × 10^{-6} | (3.49 ± 0.48) × 10^{-6} | (5.50 ± 0.74) × 10^{-4} |
| L228A | 0.25 ± 0.01 | 83.9 ± 9.24 | 137 ± 9.00 | 1.06 ± 0.10 | (3.33 ± 0.39) × 10^{-7} | (2.04 ± 0.15) × 10^{-3} | (2.64 ± 0.27) × 10^{-1} |
| S240A | 0.69 ± 0.02 | 115 ± 12.9 | 82.5 ± 5.49 | 0.38 ± 0.02 | (6.00 ± 1.22) × 10^{-4} | (8.36 ± 0.71) × 10^{-3} | 1.92 ± 0.32 |

**DISCUSSION**

The properties of Q224M, Q224E, and Q34E mutants of AMPSase can be understood in relation to the crystal structure of the IMP-NO₃ complex (19). For the wild-type enzyme (Fig. 4A), Asp13 and Gln224 putatively work in concert to stabilize the 6-oxyanion of IMP. Donor-acceptor distances between relevant atoms of Gln34, Gln224, Asp13, and IMP are consistent with the hydrogen bonding of Fig. 4A. Furthermore, a D13A mutant has no observed activity (35), consistent with its proposed role as a catalytic base.

The Q34E mutant could disrupt the hydrogen bond between Gln224 and O-6 of IMP by enforcing a conformational change on Gln224 (Fig. 4B). However, there is some uncertainty in this interpretation because the Q34E mutant has no effect on \( K_m^{\text{GTP}} \) but does significantly increase \( K_m^{\text{IMP}} \). Thus, the decrease in \( k_{cat} \) observed for the Q34E mutant may stem from conformational changes in addition to those represented in Fig. 4B.

As the \( K_m \) values for substrates are not significantly perturbed by the mutation Q224M, the decrease in \( k_{cat} \) most likely emanates from the loss of hydrogen bonds to N-7 and O-6 of IMP, the latter probably having the largest impact on catalysis (Fig. 4C). The greater reduction in \( k_{cat} \) for the Q224E mutant than for the Q224M mutant may stem from the localization of a negative charge in proximity to O-6 of IMP (Fig. 4D). Partial restoration of activity at pH 6.2, then, is due putatively to the protonation of Gln224, which reforms the critical hydrogen bond to O-6 of IMP (Fig. 4D). The failure to restore the Q224E mutant to 100% of the wild-type enzyme activity at pH 6.2 may be a consequence of (i) tautomeric states that put the proton onto O-6 (Fig. 4E) and/or (ii) the absence of the N-7 to Gln224 hydrogen bond, an absence that may undermine the stability of the hydrogen bond between Gln224 and O-6 of IMP.

The physiological importance of Gln224 is supported further by the phenomenon of reduced cell proliferation in combination with enhanced protein expression for the mutant Q224E. Mutation of essential catalytic residues severely inhibits the growth of *E. coli* in the case of D13A and H41N mutants (35). The kinetic data imply that a disruption of the purine nucleotide pathway may impair the reproductive capacity of the organism. The enhanced level of expression of the Q224E mutant may be a self-rescuing strategy of *E. coli*, to compensate for the...
impaired AMPSase and to promote adenylate synthesis by the purine nucleotide salvage pathway. It is unclear, however, why this mechanism of recovery is not operative in the case of the D13A or H41N mutant. Perhaps these mutants of AMPSase impair adenylate biosynthesis so severely that *E. coli* cannot sustain transcription and translation processes for overproduction of a given protein.

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FIG. 4. Modeling of residue 224 in the IMP binding site. Hydrogen bonds are presented as dotted lines. RSP, ribose 5'-phosphate. Each dot represents one electron. The chemical structures of side chains of Asp13, Gln/Glu14, and Gln/Glu/Met224 are illustrated. A, modeling of Gln14 and Gln224 in the active site of AMPSase. B, modeling of Gln14 and Gln224 in the active site of AMPSase. C, modeling of Gln14 and Met224 in the active site of AMPSase. D, modeling of Gln14 and Glu224 in the active site of AMPSase under basic conditions. E, modeling of Gln14 and Glu224 in the active site of AMPSase under acidic conditions.
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