Ambiguities in Mapping the Active Site of a Conformationally Dynamic Enzyme by Directed Mutation

ROLE OF DYNAMICS IN STRUCTURE-FUNCTION CORRELATIONS IN ESCHERICHIA COLI ADENYLOSUCCINATE SYNTHETASE

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On the basis of ligated crystal structures, Asn21, Asn38, Thr42, and Arg419 are not involved in the chemical mechanism of adenylsuccinate synthetase from Escherichia coli, yet these residues are well conserved across species. Purified mutants (Asp21→Ala, Asn38→Ala, Asn38→Asp, Asn38→Glu, Thr42→Ala, and Arg419→Leu) were studied by kinetics, circular dichroism spectroscopy, and equilibrium ultracentrifugation. Asp21 and Arg419 are not part of the active site, yet mutations at positions 21 and 419 lower \( k_{\text{cat}} \) 20- and 10-fold, respectively. Thr42 interacts only through its backbone amide with the guanine nucleotide, yet its mutation to alanine significantly increases \( K_m \) values for all substrates. Asn38 hydrogen-bonds directly to the 5’-phosphoryl group of IMP; yet its mutation to alanine and glutamate has no effect on \( K_m \) values, but reduces \( k_{\text{cat}} \) by 100-fold. The mutation Asn38→Asp causes 10-57-fold increases in \( K_m \) for all substrates along with a 30-fold decrease in \( k_{\text{cat}} \). At pH 5.6, however, the Asn38→Asp mutant is more active, yet binds IMP 100-fold more weakly, than the wild-type enzyme. Proposed mechanisms of ligand-induced conformational change and subunit aggregation can account for the properties of mutant enzymes reported here. The results underscore the difficulty of using directed mutations alone as a means of mapping the active site of an enzyme.

Adenylosuccinate synthetase (IMP: L-aspartate ligase (GDP-forming); EC 6.3.4.4, AMPsase) is an essential enzyme in most organisms (for review, see Ref. 1), catalyzing the first committed step in the biosynthesis of AMP.

\[
\text{IMP} + L-\text{aspartate} + GTP \leftrightarrow \text{adenylsuccinate} + \text{GDP} + \text{phosphate} \\
\text{REACTION 1}
\]

Primary sequences of AMPsase (2–10) are 40% identical for any pairwise comparison, indicating a strong tendency to preserve a primordial gene throughout evolution (10). The enzyme putatively facilitates the formation of 6-phosphoryl-IMP by the nucleophilic attack of the 6-oxo group of IMP on \( \gamma \)-phosphate of GTP, and then the formation of adenylosuccinate by displacement of the 6-phosphoryl group by \( L \)-aspartate (11, 12). AMP-Sase from Escherichia coli is a monomer at physiological concentrations (1 \( \mu \)M), but dimerizes when nucleotide ligands are present (13, 14). The dimer is probably the physiologically active form of the enzyme, having a \( K_m \) corresponding to intracellular concentrations of IMP.

The disordered active site of unligated AMP-Sase becomes ordered in the presence of substrates and substrate analogs (15–20). The largest conformational change is a 9-Å movement of the loop 42–53 (40s loop), which folds against the guanine nucleotide. Loop 120–131 (120s loop), which interacts with IMP, and loop 299–303 (300s loop), which interacts primarily with analogs of \( L \)-aspartate in crystal structures, become ordered in the presence of ligands. The conformational changes above putatively exemplify induced fit, a concept introduced by Koshland some three decades ago (21). The ligand-enzyme interactions, however, which contribute most (in terms of a thermodynamic driving force) to the observed conformational changes have yet to be identified, nor can we exclude the possibility of energy contributions from interactions between protein residues well removed from the active site.

On the basis of ligated crystal structures of adenylosuccinate synthetase, Asp21, Asn38, Thr42, and Arg419 do not interact with atoms of substrates involved with the chemistry of phototransfer or nucleophilic attack by \( L \)-aspartate (17, 19). In fact, Asp21 and Arg419 do not interact with ligands (16, 17, 19, 20). The backbone amide of Thr42 forms a hydrogen bond with the \( \alpha \)-phosphoryl group of the guanine nucleotide, but its side chain forms only a weak hydrogen bond to that same \( \alpha \)-phosphoryl group (20). Asn38 provides one of five hydrogen bonds to the \( 5' \)-phosphoryl group of IMP; implicating Asn38 in the ground-state stabilization of the IMP-enzyme complex (17, 19). Nevertheless, mutations of Asn38 have little effect on the \( K_m \) of IMP, but a major impact on \( k_{\text{cat}} \); mutations of Asp21 and Arg419 destabilize the transition state, and the mutation of Thr42 to alanine reduces affinities for all substrates. The effects of each of the mutations above can be understood in terms of their influence upon two interdependent, dynamic mechanisms in AMPsase: (i) ligand-induced reorganization of the active site and (ii) ligand-induced dimerization of the enzyme. The above mutations also illustrate the importance of a sound understanding of structure and dynamics of an enzyme before assigning functional roles to side chains.

EXPERIMENTAL PROCEDURES

Materials—GTP, IMP, \( L \)-aspartate, phenylmethylsulfonyl fluoride, and bovine serum albumin were from Sigma. Restriction enzymes were from Promega. \( Pfu \) DNA polymerase and \( E. coli \) strain XL-1 blue were obtained from Stratagene. \( E. coli \) strain H1238 (pura-) was a gift from Dr. D. Bachman (Genetic Center, Yale University, New Haven, CT). Phenyl-Sepharose CL-4B came from Amersham Pharmacia Biotech.
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Other reagents and chemicals came from Sigma if not otherwise specified.

Overexpression of Wild-type and Mutant AMPSases—Owing to the undetectable levels of expression for some mutants using the PMS204 plasmid expression system, we used here a more efficient prokaryotic expression system, based on the pTrc99A vector (Amersham Pharmacia Biotech).

The purA gene was amplified by polymerase chain reaction using the following primers to incorporate NcoI and PstI restriction sites at either end of the purA gene.

TrcN: 5′-GATGCGCATGTAACACAGCGCCG-3′ (NcoI site underlined)
TrcC: 5′-AACCTGCGTGCCGACGGTCAC-3′ (PstI site underlined)

The NcoI restriction site was incorporated into the N terminus of the purA gene, before the first Met codon (ATG), and the PstI restriction site was introduced after the stop codon.

The amplified and subcloned purA gene containing the 1.3-kilobase pair fragment was sequenced twice in both directions using the chain termination method (22) at the Nucleic Acid Facility, Iowa State University. Sequence analysis showed 100% identity with the E. coli purA sequence deposited in GenBank™, except at position 415, a GAT (Asp) is replaced by GGT (Gly), which agrees with published crystal structures (15). The purA gene was subcloned into the pTrc99A vector and the final construct, pTrpA, was transformed into E. coli strain H1238 (purA) for expression.

Site-directed Mutagenesis—The NcoI-PstI fragment containing purA was subcloned in to the pAlterEXII vector (Promega, Inc.), Site-directed mutagenesis was carried out according to published procedures from Promega, Inc. The primers used in mutations were 5′-GTAAGATCGCTGCTCTCTGAACTGA-3′ (Asp23 → Ala), 5′-GCCAGGTACGCTGGCCAATA-3′ (Asn28 → Ala), 5′-GGCCGCTCAAGCCGGGCTCA-3′ (Asn28 → Asp), 5′-GGCCGCTCAAGCCGGGCTCA-3′ (Asn28 → Glu), and 5′-CCGAGATCCGTTGCTGAACTGA-3′ (Thr42 → Ala), and 5′-CCGAGATCCGTTGCTGAACTGA-3′ (Arg419 → Leu), where the underlined bases are mismatched with respect to the wild-type purA sequence. The primer 5′-AACACCGTCGCTGCTGACTGGG-3′ was used to sequence the plasmids and confirm mutations for all but Arg419 → Leu, where the primer 5′-AACACCGTCGCTGCTGACTGGG-3′ was used to sequence the plasmids and confirm mutations for all but Arg419 → Leu.

All primers were synthesized on a Biosearch 8570EX automated DNA synthesizer at the Nucleic Acid Facility at Iowa State University. After mutagenesis, the NcoI-PstI fragment containing the desired mutation was subcloned into pTrc99A and transformed into the E. coli strain H1238 (purA) for expression.

Preparation and Kinetics of Wild-type and Mutant AMPSases—The wild-type and mutant enzymes were purified as described elsewhere (23–25) with the following modifications. The enzyme was eluted from a phenyl-Sepharose CL-4B column by a series of buffer solutions, 0.6 M, 0.4 M, and 0.2 M in (NH4)2SO4 and 50 mM in potassium Pi (pH 7.0), then further purified using a DEAETSK high performance liquid chromatography column. Enzyme purity was monitored by SDS-polyacrylamide gel electrophoresis (26), and protein concentrations were determined using Bradford reagent (Bio-Rad) (27). All the mutant enzymes were subjected to circular dichroism analysis as described elsewhere (22–24).

Directed Mutations of Adenylosuccinate Synthetase—16001

Table I

| Source               | Sequences          |
|----------------------|--------------------|
| 20–22                | 37–43              |
| 418–420              |                    |
| Bacillus subtilis    | TDF                | WR6               |
| Brucella abortus     | VDW                | WR6               |
| Dictyostelium discoideum | VDI         | WR6               |
| Escherichia coli     | VDI                | WR6               |
| Haemophilus influenzae | VDI             | WR6               |
| Homo sapiens         | VDI                | WR6               |
| Mus musculus         | VDI                | WR6               |
| Pyrococcus species   | IAY                | WR6               |
| Saccharomyces cerevisiae | VDI         | WR6               |
| Schizosaccharomyces pombe | VDI       | WR6               |
| Spirulina citri      | TDY                | WR6               |
| Thiothrix ferrooxidans | VDI               | WR6               |
| Vibrio parahydrolyticus | VDI              | WR6               |

RESULTS

Sequence Comparison and Crystallographic Analysis—Positions corresponding to Asp23, Asn28, His41, Thr42, and Arg419 are almost 100% identical across species (Table I). In the absence of ligands, Asn28 has no specific interactions, but in the ligated synthetase it hydrogen-bonds with the 5′-phosphate of IMP (Fig. 2; Ref. 17). His41, a putative catalytic acid (17, 28), has three mutually exclusive sets of interactions. (i) In the unligated synthetase, His41 hydrogen-bonds with Asp23 (16, 17). (ii) In the complex of GDP, NO3−, IMP, Mg2+, and hadacin, His41 hydrogen-bonds with the β-phosphoryl group of GDP (17). (iii) In the complex of GDP, 6-thiophosphoryl-IMP, Mg2+, and...
and hadacidin, His41 hydrogen-bonds with the 6-thiophosphoryl group and to Glu221 (19). Asp21 in the unligated synthetase hydrogen-bonds with His41, as noted above, but also makes a salt link with Arg419 in ligated complexes (17). Thr42 has no specific interaction in the unligated synthetase (16, 17), but in ligated complexes its backbone amide interacts with the α-phosphoryl group of GDP and its backbone carbonyl hydrogen-bonds with a water molecule, which in turn hydrogen-bonds with the 2′-OH of GDP and backbone carbonyl 417. In one of three ligated complexes of the synthetase (17, 19, 20), the side chain of Thr42 may interact weakly with the α-phosphate of GDP (oxygen to oxygen, donor-acceptor distance of approximately 3.1 Å; Ref. 20). The interactions above are summarized in Table II.

### Table II

| Residue | Interacting residue without ligands | Interacting residue with ligands |
|---------|-----------------------------------|----------------------------------|
| Asp21   | His41                             | Arg419 5′-Phosphoryl of IMP       |
| Asn38   |                                   | Asp21 β-Phosphoryl of GDP or 6-thiophosphoryl of 6-thiophosphoryl IMP |
| His41   |                                   |                                 |
| Thr42   |                                   |                                 |
| Backbone amide 42 |                         |                                 |
| Backbone carbonyl 417 |                         | Arg419 backbone carbonyl 42 (via a bridging water) |
| Arg419  |                                   | Asp21                                  |

Values of the Asp21 → Ala, Asn38 → Ala, and Asn38 → Glu mutants are comparable to those of wild-type AMPSase (Table III). Although $k_{cat}$ for the Thr42 → Ala mutant was the same as that of the wild-type enzyme, $K_m$ values showed 5–10-fold increases. The mutation of Asp21 to alanine reduced $k_{cat}$ 20-fold and slightly increased $K_m^{GTP}$ and $K_m^{IMP}$, whereas Arg419 reduced $k_{cat}$ 10-fold, with a slight increase in $K_m^{GTP}$, and 8-fold increases in $K_m^{GTP}$ and $K_m^{IMP}$. Asn38 → Ala, Asn38 → Asp, and Asn38 → Glu mutants showed 30–200-fold reductions in $k_{cat}$ relative to the wild-type enzyme. Of the three position Asn38 mutants, only Asn38 → Asp exhibited increased $K_m$ values ($K_m^{IMP}$ increased 80-fold).

**pH-dependent Kinetic Studies of Wild-type and Asn38 → Asp**—Of the three position 38 mutants, only $K_m$ values for the Asn38 → Asp mutant show large increases. Altered kinetic parameters for the Asn38 → Asp mutant could originate from electrostatic repulsion between the 5′-phosphoryl group of IMP and the side chain of Asp38. Protonation of the Asn38 side chain, or the 5′-phosphoryl group of IMP, then, could restore $k_{cat}$ and $K_m$ to wild-type levels. $k_{cat}$ versus pH profiles for the wild-type and the Asn38 → Asp enzymes are bell-shaped (Fig. 3). The optimum pH values for wild-type and Asn38 → Asp enzymes are 7.8 and 5.6, respectively. In fact, the Asn38 → Asp mutant had higher catalytic activity than the wild-type enzyme at pH 5.6 (Table III) and 5.2 (data not shown). Although wild-type levels of activity were recovered in the Asn38 → Asp mutant by dropping the pH, $K_m^{IMP}$ remains 100-fold higher than that of the wild-type enzyme.

**Analytic Equilibrium Ultracentrifugation**—IMP induces dimerization of AMPSase monomers (13, 14). Furthermore, the dimer arguably has 100-fold higher affinity for IMP than the monomer. Hence, we examined the Asn38 → Asp mutant by
ultracentrifugation methods, in order to determine whether its elevated $K_m^\text{GTP}$ originated from its properties of aggregation. In centrifugation studies reported here, the wild-type and Asn$^{38}$ → Glu enzymes are controls, as each exhibits comparable $K_m$ values for IMP, and hence should be dimers in the presence of IMP. The monomer-dimer dissociation constants for the three enzymes in the absence and presence of ligands at pH 7.7 are in Table IV. At pH 5.6, the three enzymes precipitated during ultracentrifugation runs. The three AMPSases show similar $K_D$ values in the absence of ligands. In the presence of ligands, however, the $K_D$ of the Asn$^{38}$ → Asp mutant (252 μM) differs significantly from that of wild-type and Asn$^{38}$ → Glu enzymes, both of which are vanishingly small (Table IV). Furthermore, the dissociation constant for Asn$^{38}$ → Asp in the presence of ligands is 19 times higher than the dissociation constant for Asn$^{38}$ → Asp in the absence of ligands.

**DISCUSSION**

For the rapid equilibrium random mechanism of AMPSase (1), $k_{\text{cat}}$ represents the breakdown of the quaternary complex of enzyme and substrates to enzyme and products. Thus, $k_{\text{cat}}$ for AMPSase is sensitive to the energy changes in the transition state. Kinetic data indicate destabilization of the transition state for the Asp$^{21}$ → Ala and Arg$^{419}$ → Leu mutants ($k_{\text{cat}}$ falls 20- and 10-fold, respectively, relative to the wild-type enzyme).

Thus, the Asp$^{21}$-Arg$^{419}$ salt link must stabilize the transition state. As the hydrogen bond between Asp$^{21}$ and Arg$^{419}$ is about 13 Å from the bound Mg$^{2+}$ (approximate center of catalysis), the stabilization is necessarily a result of an indirect mechanism. The loss of the Asp$^{21}$-Arg$^{419}$ salt link could destabilize interactions between the 40s and 400s loop. Indeed, in the unligated enzyme Arg$^{419}$ is disordered (15, 16) and the 40s and 400s loop do not interact. In the ligated enzyme, backbone carbonyl 417 hydrogen-bonds with a water molecule, which in turn hydrogen-bonds to backbone carbonyl 42 and the 2’-OH of GDP (17).

An alternative mechanism by which the loss of the Asp$^{21}$-Arg$^{419}$ salt link could influence the transition state is by way of a small perturbation of the P-loop (residues 8–16). Asp$^{13}$ is a putative catalytic base (17, 28). A displacement of its side chain by perhaps as little as 0.5 Å could lead to complete inactivation of the synthetase. The interaction of Asp$^{21}$ with Arg$^{419}$ could stabilize the position of helix H1, which lies on the C-terminal side of the P-loop. Mutations in the P-loop have only modest effects on $K_m^\text{IMP}$ and no effect on the $K_m$ for other substrates (28), consistent with the lack of change in $K_m$ values for the Asp$^{21}$ → Ala mutant.

The mutation of Arg$^{419}$ to leucine has a minor effect on the $K_m$ values for IMP and GTP. The increased $K_m^\text{GTP}$ must be a result of a localized change in the 400s loop as the Asp$^{21}$ mutation has no effect on $K_m$ values. The mutation of Arg$^{419}$ could perturb the stacking of Pro$^{417}$ with the guanine base and hence lead to the increase in $K_m^\text{GTP}$. Given the observed binding synergism of IMP and GTP (19), a reduced GTP interaction should weaken IMP binding.

The side chain of Thr$^{42}$ binds weakly (at best) to the o-phosphoryl group of GTP, yet it is a conserved residue and its mutation to alanine increases $K_m$ values by approximately 10-fold. The above phenomenon may be a consequence of the 40s loop, which is in an equilibrium between two conformational states. The “open” conformer predominates in the absence of ligands, whereas the “closed” conformer appears when ligands are bound to the active site. The absence of an appreciable effect on $k_{\text{cat}}$ caused by the mutation of Thr$^{42}$ to alanine implies no significant perturbation of the 40s loop in the ligated

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**TABLE III**

| AMPSase  | pH   | $k_{\text{cat}}$ | $K_m^\text{GTP}$ | $K_m^\text{IMP}$ | $K_m^\text{GTP}$ | $k_{\text{cat}}/K_m^\text{GTP}$ | $k_{\text{cat}}/K_m^\text{IMP}$ | $k_{\text{cat}}/K_m^\text{GTP}$ |
|---------|------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Wild-type | 7.7  | 1.00 ± 0.05     | 26 ± 2          | 28 ± 1          | 0.23 ± 0.04     | 38 ± 4          | 4.4 ± 0.8       |
|         | 5.6  | 0.996 ± 0.008   | 11 ± 1          | 70 ± 20         | 0.9 ± 0.2       | 9 ± 1           | 1.4 ± 0.4       | 0.11 ± 0.03     |
| Asp$^{21}$ → Ala | 7.7  | 0.049 ± 0.001   | 52 ± 5          | 48 ± 4          | 0.17 ± 0.01     | 0.95 ± 0.09     | 1.02 ± 0.08     | 0.30 ± 0.02     |
| Arg$^{419}$ → Leu | 7.7  | 0.100 ± 0.002   | 250 ± 20        | 200 ± 10        | 0.34 ± 0.07     | 0.41 ± 0.03     | 0.49 ± 0.03     | 0.29 ± 0.06     |
| Asn$^{38}$ → Ala | 7.7  | 0.095 ± 0.002    | 45 ± 5          | 49 ± 7          | 0.30 ± 0.02     | 0.12 ± 0.02     | 0.10 ± 0.01     | 0.016 ± 0.001   |
| Asn$^{38}$ → Asp | 7.7  | 0.034 ± 0.0003   | 118 ± 10        | 380 ± 400       | 0.5 ± 0.1       | 0.15 ± 0.2      | 0.045 ± 0.002   | 0.33 ± 0.09     |
| Asn$^{38}$ → Glu | 7.7  | 0.034 ± 0.0001   | 54 ± 5          | 28 ± 1          | 0.24 ± 0.02     | 0.62 ± 0.06     | 1.22 ± 0.06     | 0.143 ± 0.01    |
| His$^{41}$ → Asn$^a$ | 7.7  | 0.0095 ± 0.0001 | 130 ± 10        | 400 ± 8         | 2.0 ± 0.6       | 0.076 ± 0.008   | 0.024 ± 0.002   | 0.005 ± 0.002   |
| Thr$^{42}$ → Ala | 7.7  | 0.890 ± 0.02     | 280 ± 20        | 123 ± 5         | 1.4 ± 0.1       | 3.2 ± 0.2       | 7.2 ± 0.3       | 0.65 ± 0.05     |

$^a$ From Ref. 29.

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**FIG. 3.** $k_{\text{cat}}$ versus pH profiles of wild-type and Asn$^{38}$ → Asp mutant AMPSase. The $k_{\text{cat}}$ values for both enzymes were normalized for clarity of comparison. The maximum $k_{\text{cat}}$ value is 1.59 s$^{-1}$ for the wild-type enzyme at pH 7.8, and 0.237 s$^{-1}$ for Asn$^{38}$ → Asp mutant enzyme at pH 5.6.

**TABLE IV**

| AMPSase | Absence of ligands | Presence of ligands |
|---------|-------------------|---------------------|
|         | $K_m$             | Goodness of fit$^a$ | $K_m$             | Goodness of fit$^a$ |
|         | μM                |                      | μM                |                      |
| Wild-type | 15 ± 2            | 0.0009               | 0 ± 0.00006       | 0.2287               |
| Asn$^{38}$ → Asp | 14 ± 2            | 0.6014               | 250 ± 70          | 1.130                |
| Asn$^{38}$ → Glu | 24 ± 4            | 2.187                | 0 ± 0.0005        | 3.278                |

$^a$ Goodness of fit determined by the chi-square test (29).
conformation of the synthetase. If, however, the mutation to alanine stabilizes the open conformation of the loop, then ligands must spend a greater fraction of their binding energy to drive the 40s loop to its closed conformation. That the $K_m$ values increase for all substrates is a reflection of synergism in substrate binding (13).

Asn$^{38} \rightarrow$ Ala and Asn$^{38} \rightarrow$ Glu have no influence on $K_m$ values but reduce $k_{\text{cat}}$ by 200- and 30-fold, respectively. Evidently, the interaction between Asn$^{38}$ and the 5'-phosphate of IMP does not enhance the affinity of IMP for the active site. Instead, the binding energy probably is diverted completely to the stabilization of the transition state. Asn$^{38}$ belongs to a short element (residues 38-41) that immediately precedes the 40s loop. Within this structural element, the peptide link between residues 40 and 41 undergoes a significant change in conformation (17) and the $\alpha$-carbon and side-chain of Asn$^{38}$ move toward the 5'-phosphate by 1 Å. These small conformational adjustments preceding the 40s loop are probably coupled to the 9-Å movement of the loop itself. Hence, the energy of interaction of Asn$^{38}$ with the 5'-phosphate of IMP may go to support the conformational change in the 40s loop.

The mutation of Asn$^{38}$ to aspartate presents a far more complex phenomenon. The introduction of a negative charge at position 38 probably elevates $K_m^{\text{IMP}}$ by charge repulsion. All of the allowable conformations of Asp$^{38}$ bring its side chain close to the 5'-phosphate of IMP. (In contrast, allowable conformations of Glu$^{38}$ are possible, which bring about significant separation of the 5'-phosphate and the side chain.) By protonation of Asp$^{38}$ and/or the 5'-phosphate of IMP, a hydrogen bond is possible in the Asn$^{38} \rightarrow$ Asp mutant. Thus, $k_{\text{cat}}$ for the mutant at pH 5.6 is restored to wild-type levels, possibly because IMP can again drive the required conformational change in the 40s loop.

$K_m$ values for the Asn$^{38}$ $\rightarrow$ Asp mutant are not restored to wild-type levels at low pH, however, suggesting that a pH-independent mechanism is responsible for the elevated $K_m$ values of the mutant. The wild-type enzyme is predominantly a monomer in the absence of ligands at concentrations used in assays (13). Furthermore, IMP induces dimerization of the wild-type enzyme at pH 7.7, its interaction with Arg$^{143}$ of a second monomer being essential in stabilizing the dimer. The dissociation constants for Asn$^{38}$ mutant at pH 7.7 then may reflect the weak interaction of IMP and enzyme (13) and the $K_m^{\text{IMP}}$ values for the Asn$^{38}$ $\rightarrow$ Asp mutant at pH 7.7 then may reflect the weak interaction of IMP with the mutant as a monomer, rather than the binding of IMP to a mutant dimer. This is supported by the monomer-dimer dissociation constants for Asn$^{38}$ $\rightarrow$ Asp with and without ligands. The higher dissociation constant for the Asn$^{38}$ $\rightarrow$ Asp in the presence relative to the absence of ligands shows that Asn$^{38}$ $\rightarrow$ Asp favors a dimeric state in the absence of ligands. At pH 5.6, IMP may bind to the active site of the mutant as a monomer, allowing formation of the hydrogen bond with the side chain of Asp$^{38}$. However, the monomeric state of IMP may not stabilize sufficiently its interaction with Arg$^{143}$ of a second subunit. Hence, the affinity for IMP remains low, because the mutant remains a monomer at low pH. The wild-type enzyme, on the other hand, selects the dianion state of IMP from solution at low pH and, as a consequence, dimerizes. Hence, the $K_m^{\text{IMP}}$ for wild-type enzyme at pH 5.6 reflects the interaction of IMP with a synthetase dimer.

AMPase is rife with ligand-induced changes, which in a broad perspective influence subunit dimerization and/or reorganization of its active site. As a consequence of the coupling of ligand-binding energy to dynamic processes that influence the conformation of loci remote from the active site, residues can bind directly to substrates and have no effect on binding affinity as measured by $K_m$, and residues remote from the active site can have substantial effects on the stability of the transition state or the binding affinity of substrates. Such phenomena underscore the pitfalls in using site directed mutations alone in assigning residues to the active site of an enzyme.

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