Allosteric and Catalytic Functions of the PP<sub>i</sub>-binding Motif in the ATP Sulfurylase-GTPase System*

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ATP sulfurylase, from Escherichia coli K-12, catalyzes and couples the Gibbs potentials of two reactions, GTP hydrolysis and activated sulfate (APS, adenosine 5'-phosphosulfate) synthesis. Coupling these potentials requires that the catalytic cycle include reaction stage-dependent conformational changes that gate the activities of the two active sites. These interactions were probed in a mutagenesis study of a highly conserved pyrophosphate-binding motif (SXGXDS), which is located at the APS-forming active site. The motif appears to be unique to the N-type PP<sub>i</sub>, synthetase family, and mutations in it are linked, in other systems, to citrullinemia, an often fatal orphan disease. The conserved sites in the motif were evaluated individually for their ability to activate GTP hydrolysis (which reports interactions among the activator (AMP or Mg<sup>2+</sup>·PP<sub>i</sub>), the enzyme, and GTP), to affect the energetic coupling of the two reactions, and to alter the kinetic constants of the adenylyl transfer reaction in the absence of guanine nucleotide. What emerges from this first mutagenic exploration of the PP<sub>i</sub> motif in any adenylyltransferase is that the residues of the motif participate differently, and in sometimes profoundly important ways, in the different functions of the enzyme.

ATP sulfurylase, from Escherichia coli K-12, is a rare example of a GTPase-coupled system in which the conformational events usually involved in target interactions are used, instead, to link the Gibbs potential of GTP hydrolysis to another small molecule reaction, the synthesis of activated sulfate (adenosine 5'-phosphosulfate, or APS), as shown in Reactions 1 and 2 (1, 2).

\[ \text{ATP}^4+ + \text{SO}_4^{2-} \rightleftharpoons \text{APS}^{2-} + \text{P}_2\text{O}_7^{4-} \]

**REACTION 1**

\[ \text{GTP}^4+ + \text{H}_2\text{O} \rightleftharpoons \text{GDP}^{3+} + \text{HPO}_4^{2-} + \text{H}^+ \]

**REACTION 2**

The native enzyme is a tetramer of heterodimers, each of which is composed of a GTPase subunit (CysN, 53 kDa) and an adenylyltransferase subunit (CysD, 23 kDa) (3, 4). ATP sulfurylase links these two chemistries with high efficiency (1:1 stoichiometry) and couples virtually all of the chemical potential available in the hydrolysis reaction to APS synthesis (1). The phosphoric-sulfuric acid anhydride bond formed during the transfer reaction chemically activates the otherwise non-reactive sulfate for participation in its subsequent metabolic biochemistry: reduction and sulfuryl transfer.

Activated sulfate is essential for sulfur redox metabolism in bacteria (5). Certain organisms, including E. coli, reduce sulfate to sulfide, which is needed for the biosynthesis of reduced sulfur metabolites. Others use this reduction to draw electrons from their electron transport chains, whereas their symbionts oxidize reduced sulfur to provide electrons to these chains. Mammals use activated sulfate for an entirely different purpose, sulfuryl transfer. This reaction is used widely by the cell to regulate important metabolic processes including steroid and peptide hormone action (6–8), hemostasis (9–13), lymph cell circulation (14, 15), and growth factor recognition (16).

As ATP sulfurylase moves through the various stages of its catalytic cycle, the ground and transition state energetics of the GTPase activity are modulated by inorganic pyrophosphate in ways that depend upon the role that PP<sub>i</sub> is asked to play. When it is the sole activator of GTP hydrolysis, PP<sub>i</sub> stimulates GTPase turnover 20-fold over the basal or non-activated level (0.010 s<sup>-1</sup> (17)), and the interaction energies that accelerate catalysis occur predominantly downstream of the partially rate-limiting isomerization that precedes scission of the β,γ-bond (18–21). When acting in concert with AMP to activate hydrolysis, PP<sub>i</sub> shifts the system 60-fold further toward the isomerized/hydrlytic form, and its release contributes to a resetting of the enzyme to its non-isomerized state. Identifying how and where the reaction coordinate is influenced by the changing contacts between PP<sub>i</sub> and the enzyme is fundamental to understanding how the catalytic cycles are coupled and how catalysis is accomplished in this system.

The PP<sub>i</sub> motif, a highly conserved sequence that appears unique to N-type adenylyl transferases (22), is N-terminally located in the primary sequence of ATP sulfurylase. The motif is defined by a hydrophobic β-strand and α-helix flanking the N- and C-terminal sides of a glycine-rich consensus sequence, SXGXDS, a pattern reminiscent of the Walker A motif (23, 24). The structures of five N-type adenylyltransferases reveal that the architecture of the PP<sub>i</sub> motif and its extensive interactions with Mg<sup>2+</sup>·PP<sub>i</sub> are remarkably well conserved (see “Results and Discussion”). The functions of each of the conserved residues of the motif in adenylyl transfer and allosteric interactions between the APS-forming and GTPase active sites were explored in the current investigation through subtle mutagenic alterations that revealed that the roles of the residues change with the catalytic demands placed upon them.

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Plasmid was verified. Sub-plasmid. The full coding sequence of the amplified region of the agarose gel electrophoresis and ligated into the digested and purified digested with BamHI and XbaI. The digested insert was purified by agarose gel electrophoresis and this insert was purified by agarose gel electrophoresis and inserted into the digested insert (containing the BamHI and XbaI restriction sites) to generate the full-length mutant native plasmid) and amplified using the flanking primers encoding the native form of the enzyme was the PCR template. The left half of the insert (containing the BamHI site and the site of mutation) were separately amplified using the polymerase. These three-dimensional structure. The sequences and conserved secondary structures were identified except for slight (single position) shifts of the boundaries that separate the three structural elements (α-helix, PPi loop) of the best-match proteins are shown in Fig. 1. Three different secondary structure prediction algorithms (PROF, University of Wales; Jpred, University of Dundee; and nnPredict, University of California) predicted that the secondary structural elements conserved in the known PPi synthetase domain are also found in CysD and other classes of enzymes that have PPi synthetase activity (e.g., GMP synthetases, NAD+ synthetases, asparagine synthetases, and argininosuccinate synthetases) (22, 26–31). Using CysH as the template, proteins containing the conserved PPi synthetase domain were selected with the Vector Alignment Search Tool algorithm, VAST (National Center for Biotechnology Information). This program compares the primary sequence and structural motifs of related proteins to identify regions of conserved three-dimensional structure. The sequences and conserved secondary structures (α-helix, β-sheet, PPi loop) of the best-matching proteins are shown in Fig. 1. Three different secondary structure prediction algorithms (PROF, University of Wales; Jpred, University of Dundee; and nnPredict, University of California) predicted that the secondary structural elements conserved in the known PPi synthetase domain are also found in the putative CysD PPi synthetase domain. The predictions were identical except for slight (single position) shifts of the boundaries that separate the three structural elements (α-helix, PPi-binding loop, and β-sheet). The predicted CysD secondary structure is shown.

The nearly identical domain architecture shared by those enzymes with the highest degree of sequence homology to the CysD subunit is presented in Fig. 2A. The main chain α-carbons of GMP synthetase, NAD+ synthetase, and asparagine synthetase were aligned with GMP synthetase as the template, using Insight II (Accelrys Inc.). These three enzymes have absolute conservation of the SXGXS motif found in CysD. The standard deviations between the Ca atoms of GMP synthetase and the two over lain enzymes were very small (0.38 and 0.57 Å for NAD+ synthetase and asparagine synthetase, respectively). This domain architecture is also highly conserved in arginino-succinate synthetase and adenosine 3′-phosphate, 5′-phospho-

RESULTS AND DISCUSSION

A Model for the ATP Sulfurylase PPi Motif—The accuracy of any structural model for the ATP sulfurylase PPi motif depends upon the degree to which the ATP sulfurylase motif conforms to the consensus features of the PPi motif found in N-type adenylyl transferase for which structures are available. The Basic Local Alignment Search Tool, BLAST, retrieved E. coli CysH PAPS reductase as the protein with the greatest sequence similarity (24% sequence identity and 39% sequence homology) to the CysD alkyltransferase motif. Blastp matches with PDB structures (Swiss Institute of Bioinformatics network). CysH contains the N-type PPi synthetase sequence motif found in CysD and other classes of enzymes that have PPi synthetase activity (e.g. GMP synthetases, NAD+ synthetases, asparagine synthetases, and argininosuccinate synthetases) (22, 26–31). Using CysH as the template, proteins containing the conserved PPi synthetase domain were selected with the Vector Alignment Search Tool algorithm, VAST (National Center for Biotechnology Information). This program compares the primary sequence and structural motifs of related proteins to identify regions of conserved three-dimensional structure. The sequences and conserved secondary structures (α-helix, β-sheet, PPi loop) of the best-matching proteins are shown in Fig. 1. Three different secondary structure prediction algorithms (PROF, University of Wales; Jpred, University of Dundee; and nnPredict, University of California) predicted that the secondary structural elements conserved in the known PPi synthetase domain are also found in the putative CysD PPi synthetase domain. The predictions were identical except for slight (single position) shifts of the boundaries that separate the three structural elements (α-helix, PPi-binding loop, and β-sheet). The predicted CysD secondary structure is shown.

The nearly identical domain architecture shared by those enzymes with the highest degree of sequence homology to the CysD subunit is presented in Fig. 2A. The main chain α-carbons of GMP synthetase, NAD+ synthetase, and asparagine synthetase were aligned with GMP synthetase as the template, using Insight II (Accelrys Inc.). These three enzymes have absolute conservation of the SXGXS motif found in CysD. The standard deviations between the Ca atoms of GMP synthetase and the two overlain enzymes were very small (0.38 and 0.57 Å for NAD+ synthetase and asparagine synthetase, respectively). This domain architecture is also highly conserved in arginino-succinate synthetase and adenosine 3′-phosphate, 5′-phospho-
sulfate reductase (not shown), which do not have absolute conservation of the SXGDXS motif (see Table I). In an effort to identify the conserved contacts between PP1 and its module, PP1, from the GMP synthetase and from the active sites of all three proteins was overain, as shown in Fig. 2B. The active site structures are nearly identical. 

**The Catalytic Integrity of the Sites—Changes in GTPase activation parameters caused by mutagenic substitution at a particular position indicate that the native residue at that position is part of a molecular framework either that undergoes structural change during allosteric communication or in which the substitution impinges in some way upon that framework, altering its behavior. Such alterations range from subletlies too small to observe crystallographically to gross structural changes that obliterate function. The AMP and PP1 subsections of the APS-forming active site of ATP sulfurylase can be probed individually for their ability to bind ligand and communicate to the GTPase active site. A well functioning AMP-binding site, observed in all cases in this study (see below), demonstrates that a mutation in the PP1 motif does not disrupt structure sufficiently to affect function at the contiguous AMP-binding subsection of the same active site.

**The Binding of AMP—Pyrophosphate synthetase structures in which the \(\alpha,\beta\)-phosphate of ATP is cleaved show that direct interactions between the conserved residues of the PP1 motif and substrate are limited to interactions with \(\text{Mg}^{2+}\)-PPi; there are no direct AMP interactions (29). These well isolated contacts predict that binding interactions between the enzyme and AMP may be affected only slightly by substitutions at the conserved positions. This prediction is validated by the AMP-activation data compiled in Table I. The number above the line in each data cell in Table I is the value of the kinetic constant and its standard error; below the line, the value of the constant is given relative to that of the wild-type enzyme. Representative activation data are shown in Fig. 3, A and B. The \(K_A\) values, which describe the interaction of AMP with the enzyme in the absence of \(\text{Mg}^{2+}\)-GTP, are very similar for the wild-type and mutant enzymes; the differences are less than \(\pm 2\)-fold. Similar small differences are observed in the \(K_A\) values, which describe interactions between AMP and E-\(\text{Mg}^{2+}\)-GTP.

Mutations at three out of the four conserved positions have little effect on the steady-state affinity of GTP (changes in \(K_{G\text{TP}}\) and \(K_{m\text{GTP}}\) are less than \(2\)-fold). The exception is the D39A mutation, which decreases the steady-state affinity of \(\text{Mg}^{2+}\)-GTP for E (8-fold) and E-AMP (5-fold). Thus, the changes in the interactions between the Asp-39 carboxylate and its environment caused by the mutation are "sensed" by the GTP-binding site and regulate its affinity for GTP. In this example, the perturbation is the exchange of a proton for the carboxylate of Asp-39. However, during the catalytic cycle, changes at that position might be caused by an event(s) such as bond cleavage or migration of \(\text{Mg}^{2+}\) along the triphosphate chain. A change in the environment at that position reports that an event has occurred to the distal site, which responds, resulting, finally, in altered function at both sites. Such is the nature of the reaction stage-specific allostery that underlies the linkage of chemical potentials in this and other systems. It is notable that the GTP hydrolisis \(k_\text{cat}\) is virtually unaffected by the mutations. Thus, the activation energetics of the rate-determining events in the catalytic cycle do not appear to be linked to the changes caused by these substitutions in the absence of \(\text{Mg}^{2+}\)-PPi.

It is important to realize that these experiments probe the interactions of a residue in a binding pocket with its environment in the absence of its ligand, \(\text{Mg}^{2+}\)-PPi. The effects of mutating a residue integral to the molecular communication between the sites may be silent in the absence of ligand but emerge when binding of the allosteric "forces" an environment that discriminates between the native and mutant residues.

**The Binding of PP1—The affinities of \(\text{Mg}^{2+}\)-PP1 and \(\text{Mg}^{2+}\)-GTP for the enzyme vary markedly depending on the mutation and occupancy at the complementary site. Effects on the affinity of \(\text{Mg}^{2+}\)-PP1 for E (i.e., \(K_{m\text{mutant}}/K_{m\text{wild-type}}\)) range
from a negligible 0.9-fold effect at Ser-35 to a maximum 16-fold decrease in the D39A mutant (Table II). Effects on the interactions of the enzyme and Mg$^{2+}$-GTP are small; $K_a$(GTP) ranges from 0.5 to 3 times that of wild type. These effects are amplified at each position when the complementary ligand is docked at its active site (Table I and Fig. 4A). Thermodynamic additivity requires that destabilization caused by simultaneous occupancy of both binding sites be mutual (experienced by both ligands) and identical in magnitude. These effects are given by $K_{ia}$/$K_a$ or $K_a$(GTP)/$K_m$(GTP); moving counterclockwise around the PPi-ring, beginning with Ser-40 (Fig. 2B), the ratios are: 6.1 (S40A), 65 (D39A), 26 (G37A), and 6.7 (S35A). Thus, the binding of either ligand drives its interacting partner from the surface of the enzyme, a reversal of the native behavior, where simultaneous occupancy stabilizes binding.

Of all of the mutants studied, D39A exhibits the largest effects on ligand affinities. This mutation, in which a carboxylate is replaced by a proton, causes a small reduction in molecular volume, the loss of a negative charge, and a considerable change in bonding properties. A reduction in volume argues that the effects are more likely attributable to a loss of charge and bonding capability than to steric factors. The precise molecular basis of the effects is unknown. However, GTP binding clearly engenders an enhanced dependence on the carboxyl moiety for stable binding of Mg$^{2+}$-PPi; the enzyme has become more discriminating toward Mg$^{2+}$-PPi binding. Replacing the hydroxyl of either serine (40 or 35) with a proton reveals that these residues interact differently with the $\beta$-phosphoryl group, and the steric/electronic and selectivity issues discussed above also apply here.

The substitution at Gly-37 differs from those at the other sites in that the molecular volume is increased, rather than decreased; a Cα-proton is replaced by a methyl group. The introduction of the methyl group affects $K_a$ and $K_n$ for the PPi-activated hydrolysis of GTP (7.2- and 180-fold increase, respectively) and is the only substitution that significantly

### Table I

| Protein   | $K_{ia}$ (mM) | $K_a$ (mM) | $K_a$(GTP) (mM) | $K_m$(GTP) (mM) | $k_{cat}$ (sec$^{-1}$) |
|-----------|---------------|------------|----------------|-----------------|-------------------------|
| Wild Type | 0.49 (0.06)   | 0.22 (0.02) | 0.12 (0.01)    | 0.051 (0.003)   | 2.1 (0.05)              |
| S40A      | 0.50 (0.03)   | 0.40 (0.01) | 0.10 (0.006)   | 0.083 (0.002)   | 1.0 (0.01)              |
| D39A      | 1.03 (0.2)    | 0.28 (0.09) | 0.90 (0.41)    | 0.24 (0.04)     | 3.6 (0.2)               |
| G37A      | 0.46 (0.03)   | 0.63 (0.02) | 0.075 (0.005)  | 0.10 (0.003)    | 1.87 (0.02)             |
| S35A      | 0.68 (0.1)    | 0.57 (0.05) | 0.060 (0.01)   | 0.050 (0.005)   | 1.5 (0.05)              |
| Wild Type | 0.15 (0.04)   | 0.022 (0.002)| 0.06 (0.008)   | 0.009 (0.002)   | 0.33 (0.008)            |
| S40A      | 0.69 (0.1)    | 0.63 (0.05) | 0.18 (0.03)    | 0.17 (0.01)     | 0.25 (0.008)            |
| D39A      | 2.4 (0.3)     | 24 (3.5)    | 0.030 (0.003)  | 0.29 (0.05)     | 0.24 (0.05)             |
| G37A      | 1.0 (0.4)     | 4.0 (0.8)   | 0.029 (0.007)  | 0.11 (0.03)     | 0.035 (0.005)           |
| S35A      | 0.13 (0.06)   | 0.13 (0.03) | 0.085 (0.03)   | 0.085 (0.02)    | 0.24 (0.02)             |

\[ a \] The kinetic constant, and its standard error, are listed above the line; beneath it, the constant is normalized to that of the wild-type enzyme.

\[ b \] The ratio of the initial rates of GTP hydrolysis and ATP synthesis and its quotient are listed above the line; beneath it, the ratio is normalized to that of the wild-type enzyme.
The Adenylyl Transfer Activity—The molecular functions of the conserved PP_1 motif residues in adenylyl transfer reactions have not been assigned in any such system despite the fact that mutations that cause citrulinemia, an often fatal orphan disease, cluster in this motif in argininosuccinate synthetase (31, 32). There are several parallels between the effects of mutation on the kinetic constants for GTPase activation and adenylyl transfer. Like AMP, the steady-state affinity of APS (in this case, for E and E-PP_i) is only moderately affected by the mutations (0.5–6.5 times that of wild type), as shown in Table II and Fig. 4B. Representative initial rate data are shown in Fig. 3C. Thus, the addition of the sulfonyl moiety at _P_a-AMP does not radically alter linkage to the PP_1 site. The Mg_2^+-PP_i affinities for E and E-PP_i are remarkably different. In all cases, the binding of APS decreases Mg_2^+-PP_i affinity. The sites clearly communicate in a position-dependent fashion, a finding that is not readily appreciated by inspecting the structures of adenylyltransferases. The magnitude and position dependence of the Mg_2^+-PP_i affinity changes parallel those observed for the Mg_2^+-PP_i/Mg_2^+-GTP interactions, suggesting the possibility of similar PP_1 loop conformations in both complexes.

The effects on _k_cat for the synthesis of ATP and sulfate range widely across the conserved positions. Substitution of the hydroxyl at Ser-40 with a proton results in a 5-fold increase in _k_cat; whereas the identical substitution at Ser-35 has virtually no effect (1.4-fold). Hence, these two residues, which straddle and interact directly with the _β_-phosphate of ATP, are functionally non-equivalent. Given its small to negligible effects on every parameter tested, the Ser-35 hydroxyl appears to be the least catalytically involved of the conserved residues. Replacing the pro-i proton of Gly-37 with a methyl group reduces turnover to 8.5% that of wild type. Thus, rate-limiting steps are added.

affects turnover of the hydrolysis site (_k_cat is decreased 9-fold). Because it lacks a side chain, glycine has more energetically favorable dihedral angles available to it than other amino acids. Structures indicate that Gly-37, which is centrally positioned in the binding loop, does not interact directly with Mg_2^+-PP_i. An analysis of the consensus features of the PP_i motif in a set of 28 proteins from various species reveals that this glycine is the only absolutely conserved residue (22). It may well be that the glycine is conserved because of the importance of its dihedral flexibility in the proper functioning of the loop and that introduction of the alanine side chain restricts the backbone dihedral angles, which reorients and changes the interactions of neighboring groups that interact directly with Mg_2^+-PP_i.

The 9-fold decrease in GTP hydrolysis that occurs when the pro-i proton of Gly-37 is replaced by a methyl group is intriguing. Our previous studies indicate that turnover of the Mg_2^+-GTP-E-AMP-Mg_2^+-PP_i complex involves at least two partially rate-determining steps: an isomerization that precedes scission of the _β,γ_-bond of GTP and the release of GDP. It is reasonable, but not necessary, that this substitution affects one (or both) of these steps. Regardless of precisely where in the catalytic cycle the linkage occurs, the data clearly indicate that a rate-determining conformational change is linked to, and inhibited by, the methyl substituent, which means that Gly-37 is either part of the molecular framework that changes during the rate-determining transition or impinges upon that framework when the methyl group is added.

The ATP Sulfurylase-GTPase PP_i Motif

FIG. 3. Representative data from initial rate studies of ATP sulfurylase. Panel A, the AMP-activated hydrolysis of GTP. Reaction progress was monitored by following the UV change associated with the oxidation of NADH, which was coupled to the production of GDP using pyruvate kinase and lactate dehydrogenase. The reaction conditions were as follows: ATP sulfurylase (1.3 _μ_μ), AMP and GTP (at the concentrations indicated), pyruvate kinase (15 units/ml), lactate dehydrogenase (5.0 units/ml), HepeK^- (50 mM, pH 8.0), P-enolpyruvate (1.0 mM), NADH (0.20 mM), MgCl_2 (1.0 mM + [GTP]), temperature -25 _±_ 2 °C. Panel B, the PP_i-activated hydrolysis of GTP. Reaction progress was monitored as in Panel A. The reaction conditions were as follows: ATP sulfurylase D39A mutant (2.9 _μ_μ), PP_i and GTP (at the concentrations indicated), pyruvate kinase (15 units/ml), lactate dehydrogenase (5.0 units/ml), HepeK^- (50 mM, pH 8.0), P-enolpyruvate (1.0 mM), NADH (0.20 mM), MgCl_2 (0.70 mM + [PP_i] + [GTP]), temperature -25 _±_ 2 °C. Panel C, the ATP-forming reaction. Reaction progress was monitored by following the UV change associated with the reduction of NADP^+, which was coupled to the production of ATP using hexokinase and glucose-6-phosphate dehydrogenase. The reaction conditions were as follows: ATP sulfurylase S40A mutant (0.26 _μ_μ), APS and PP_i, (at the concentrations indicated), hexokinase (9.2 units/ml), glucose-6-phosphate dehydrogenase (0.50 units/ml), HepeK^- (50 mM, pH 8.0), glucose (10 mM), NADP^+ (0.20 mM), KCl (10 mM), MgCl_2 (1.0 mM + [PP_i]), temperature -25 _±_ 2 °C. Panel D, energy coupling efficiency of the native and mutant ATP sulfurylases. Efficiency is the ratio of the initial rate of APS formation to the initial rate of GTP hydrolysis under saturating conditions. GTP hydrolysis was monitored as described in panels A and B. The hydrolysis reaction was initiated by the addition of ATP sulfurylase to a solution containing pyrophosphatase (2.0 units/ml), pyruvate kinase (50 mM, pH 8.0), P-enolpyruvate (1.0 mM), NADP^+ (0.20 mM), MgCl_2 (0.70 mM + [GTP]), temperature -25 _±_ 2 °C. APS kinase was then added (1/200 dilution) to 0.4 units/ml, and the reaction rate was determined after the system reached steady-state. The rate of APS synthesis was calculated from the difference in rates before and after the addition of APS kinase.

A. 

B. 

C. 

D. 

Representative data from initial rate studies of ATP sulfurylase. Panel A, the AMP-activated hydrolysis of GTP. Reaction progress was monitored by following the UV change associated with the oxidation of NADH, which was coupled to the production of GDP using pyruvate kinase and lactate dehydrogenase. The reaction conditions were as follows: ATP sulfurylase (1.3 _μ_μ), AMP and GTP (at the concentrations indicated), pyruvate kinase (15 units/ml), lactate dehydrogenase (5.0 units/ml), HepeK^- (50 mM, pH 8.0), P-enolpyruvate (1.0 mM), NADH (0.20 mM), MgCl_2 (1.0 mM + [GTP]), temperature -25 _±_ 2 °C. Panel B, the PP_i-activated hydrolysis of GTP. Reaction progress was monitored as in Panel A. The reaction conditions were as follows: ATP sulfurylase D39A mutant (2.9 _μ_μ), PP_i and GTP (at the concentrations indicated), pyruvate kinase (15 units/ml), lactate dehydrogenase (5.0 units/ml), HepeK^- (50 mM, pH 8.0), P-enolpyruvate (1.0 mM), NADH (0.20 mM), MgCl_2 (0.70 mM + [PP_i] + [GTP]), temperature -25 _±_ 2 °C. Panel C, the ATP-forming reaction. Reaction progress was monitored by following the UV change associated with the reduction of NADP^+, which was coupled to the production of ATP using hexokinase and glucose-6-phosphate dehydrogenase. The reaction conditions were as follows: ATP sulfurylase S40A mutant (0.26 _μ_μ), APS and PP_i, (at the concentrations indicated), hexokinase (9.2 units/ml), glucose-6-phosphate dehydrogenase (0.50 units/ml), HepeK^- (50 mM, pH 8.0), glucose (10 mM), NADP^+ (0.20 mM), KCl (10 mM), MgCl_2 (1.0 mM + [PP_i]), temperature -25 _±_ 2 °C. Panel D, energy coupling efficiency of the native and mutant ATP sulfurylases. Efficiency is the ratio of the initial rate of APS formation to the initial rate of GTP hydrolysis under saturating conditions. GTP hydrolysis was monitored as described in panels A and B. The hydrolysis reaction was initiated by the addition of ATP sulfurylase to a solution containing pyrophosphatase (2.0 units/ml), pyruvate kinase (15 units/ml), lactate dehydrogenase (5.0 units/ml), HepeK^- (50 mM, pH 8.0), P-enolpyruvate (1.0 mM), NADP^+ (0.20 mM), MgCl_2 (1.0 mM + [ATP] + [GTP]), temperature -25 _±_ 2 °C. APS kinase was then added (1/200 dilution) to 0.4 units/ml, and the reaction rate was determined after the system reached steady-state. The rate of APS synthesis was calculated from the difference in rates before and after the addition of APS kinase.

A. 

B. 

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and/or altered by the substitution, which again raises the question of a need for torsional freedom at this absolutely conserved position. Replacing the carboxyl group of Asp-39 with a proton has a profound effect on turnover; it is reduced to less than 0.01% of the wild-type enzyme. This mutant binds both AMP and PPi, and either activator simulates GTP hydrolysis, suggesting that the catalytic cycle arrests at a point following assembly of the APS-PPi complex.

Effects on the Coupling Efficiency—The conformational coupling of the chemical potentials of the two reactions requires that the chemistries pass through conformational events that interdigitate their steps. The efficiency of these coupling events determines the degree to which the chemical potential available in one reaction is used to drive the other. Under appropriate conditions, GTP hydrolysis and APS synthesis are tightly linked in a 1:1 stoichiometry. To assess whether the conserved PPi loop residues are linked to the coupling steps of the reaction, the coupling efficiency (i.e., \( k_{\text{cat(APS synthesis)}} / k_{\text{cat(GTP hydrolysis)}} \)) of each mutant was compared with that of the wild-type enzyme (Fig. 3D and Table II). Efficiency was impaired in all cases; that is, the APS-forming reaction was slowed relative to GTP hydrolysis, which was only moderately affected. Thus, the energetic barriers that commit the chemistries to stepwise, 1:1, catalysis are eroded by the mutations such that one round of APS synthesis need not be completed during each round of the GTPase cycle; at some point(s), the reactions are uncoupled. It is important to realize that the APS detected in this assay is produced via the coupled pathway; this is true because the rate of uncoupled APS synthesis (i.e., in the absence of GTP) is negligible. Furthermore, activation of GTP hydrolysis, which occurs in all cases here, requires that the activating ligand(s) are bound at the APS-forming site and that an isomerization that precedes GTP hydrolysis occurs. Thus, the coupling remains sufficiently intact for the enzyme to bind ligand and move through the isomerization, produce APS and PPi, and complete the GTPase catalytic cycle at a net rate comparable with that of the wild-type enzyme. Identifying where in the catalytic cycle the APS-forming reaction arrests will help to pinpoint the energy coupling events in the mechanism, and to understand how these events are linked to the PPi-binding motif.

Conclusions—The roles of specific PPi motif residues in adenylate transfer and allosteric communication between the APS-forming and GTPase sites of ATP sulfurylase have been explored using mutagenesis. The PPi loop mutations are not linked to the binding of AMP. In the absence of Mg\(^{2+}\)-PPi, G37A is the only mutation that significantly affects binding at the GTPase site; effects on the hydrolysis \( k_{\text{cat}} \) are quite small. The linkage changes radically when the PPi-binding site is occupied.
pied: the effects are larger, each mutation alters the affinity of Mg\textsuperscript{2+}-PP\textsubscript{i} and Mg\textsuperscript{2+}-GTP for E, and certain mutants (D39A and G37A) exhibit large binding-destabilizing interactions when the sites are simultaneously occupied. D39A is the only mutant to show linkage to the transition state energetics of the hydrolysis reaction. The APS synthesis and GTP hydrolysis reactions are uncoupled to varying degrees by the mutations, all of which impede the APS-forming reaction relative to GTP hydrolysis, which is affected only slightly.

The effects on adenylyl transfer catalysis range widely. $k_{cat}$ ranges from a 5-fold stimulation, when compared with wild-type enzyme, to no detectable turnover despite the fact that the enzyme binds substrates and communicates with the GTPase site. The mutations only slightly influence the affinity of APS for E, whereas Mg\textsuperscript{2+}-PP\textsubscript{i} binding (in certain cases) is decreased substantially and a residue-dependent, destabilizing synergy is observed between the sites.

REFERENCES
1. Liu, C., Suo, Y., and Leyh, T. S. (1994) *Biochemistry* 33, 7309–7314
2. Leyh, T. S., and Suo, Y. (1992) *J. Biol. Chem.* 267, 542–545
3. Leyh, T. S., Taylor, J. C., and Markham, G. D. (1988) *J. Biol. Chem.* 263, 2409–2416
4. Leyh, T. S., Vogl, T. F., and Suo, Y. (1992) *J. Biol. Chem.* 267, 10405–10410
5. Odom, J. M., and Singleton, R., Jr. (ed.) (1993) The Sulfate-Reducing Bacteria: Contemporary Perspectives, pp. 1–19, Springer-Verlag, New York
6. Falany, C. N., Wheeler, J., Oh, T. S., and Falany, J. L. (1994) *J. Steroid Biochem.* 48, 369–375
7. Pasqualini, J. R., Schatz, B., Varin, C., and Nguyen, B. L. (1992) *J. Steroid Biochem.* 41, 223–229
8. Brand, S. J., Andersen, B. N., and Rehfeld, J. F. (1984) *Nature* 309, 456–458
9. Leyh, T. S. (1993) *CRC Crit. Rev. Biochem. Mol. Biol.* 28, 515–542
10. Leyth, A., van Schijndel, H. B., Niehrs, C., Huttner, W. B., Verheet, M. P., Mertens, K., and van Mourik, J. A. (1991) *J. Biol. Chem.* 266, 740–746
11. Stone, R. S., and Hofsteenge, J. (1986) *Biochemistry* 25, 4622–4628
12. Tollefsen, D. M., Majerus, D. W., and Blank, M. K. (1982) *J. Biol. Chem.* 257, 2162–2169
13. Van Deerlin, V. M., and Tollefsen, D. M. (1991) *J. Biol. Chem.* 266, 20223–20231
14. Hortin, G. L., Farries, T. C., Graham, J. P., and Atkinson, J. P. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 1338–1342
15. Hemmerich, S., Bertozi, C. R., Leffler, H., and Rosen, S. D. (1994) *Biochemistry* 33, 4520–4529
16. Ishihara, M., Guo, Y., and Swiedler, S. J. (1993) *Glycobiology* 3, 83–88
17. Wang, R., Liu, C., and Leyh, T. S. (1995) *Biochemistry* 34, 490–495
18. Wei, J., and Leyh, T. S. (1998) *Biochemistry* 37, 17163–17169
19. Wei, J., and Leyh, T. S. (1999) *Biochemistry* 38, 6311–6316
20. Yang, M., and Leyh, T. S. (1997) *Biochemistry* 36, 3270–3277
21. Sukal, S., and Leyh, T. S. (2001) *Biochemistry* 40, 15009–15016
22. Bork, P., and Koonin, E. V. (1994) *Proteins* 20, 347–355
23. Walker, J. E., Saraste, M., Runwick, M. J., and Gay, N. J. (1982) *EMBO J.* 1, 945–951
24. Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990) *Trends Biochem. Sci.* 15, 430–434
25. Wei, J., Tang, Q. X., Varlamova, O., Roche, C., Lee, R., and Leyh, T. S. (2002) *Biochemistry* 41, 8493–8498
26. Nessi, M., Albertini, A. M., Speranza, M. L., and Galirzi, A. (1995) *J. Biol. Chem.* 270, 6181–6185
27. Rizzi, M., Nessi, C., Mattevi, A., Coda, A., Bolognesi, M., and Galirzi, A. (1996) *EMBO J.* 15, 5125–5134
28. Savage, H., Montoya, G., Svensson, C., Schwenn, J. D., and Sinning, I. (1997) *Structure* 5, 895–896
29. Tesmer, J. J., Klem, T. J., Deras, M. L., Davisson, V. J., and Smith, J. L. (1997) *Nat. Struct. Biol.* 3, 74–80
30. Laroren, R. M., Boehrlein, S. K., Schuster, S. M., Richards, N. G., Thoden, J. B., Holden, H. M., and Rayment, I. (1999) *Biochemistry* 38, 6181–6185
31. Lemke, C. T., and Howell, P. L. (2001) *Structure (Camb.)* 9, 1153–1164
32. Nagata, N., Matsuda, I., and Oyanagi, K. (1991) *Am. J. Med. Genet.* 39, 228–229
33. Cleland, W. W. (1979) *Methods Enzymol.* 63, 103–138