PurT-encoded Glycinamide Ribonucleotide Transformylase

ACCOMMODATION OF ADENOSINE NUCLEOTIDE ANALOGS WITHIN THE ACTIVE SITEx

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PurT-encoded glycinamide ribonucleotide transformylase, or PurT transformylase, functions in purine biosynthesis by catalyzing the formulation of glycinamide ribonucleotide through a catalytic mechanism requiring Mg²⁺-ATP and formate. From previous x-ray diffraction analyses, it has been demonstrated that PurT transformylase from *Escherichia coli* belongs to the ATP-grasp superfamily of enzymes, which are characterized by three structural motifs referred to as the A-, B-, and C-domains. In all of the ATP-grasp enzymes studied to date, the adenosine nucleotide ligands are invariably wedged between the B- and C-domains, and in some cases, such as biotin carboxylase and carbamoyl phosphate synthetase, the B-domains move significantly upon nucleotide binding. Here we present a systematic and high-resolution structural investigation of PurT transformylase complexed with various adenosine nucleotides or nucleotide analogs including Mg²⁺-ATP, Mg²⁺-5′-adenylylimidodiphosphate, Mg²⁺-β,γ-methyleneadenosine 5′-triphosphate, Mg²⁺-ATPγS, or Mg²⁺-ADP. Taken together, these studies indicate that the conformation of the so-called "P-loop," delineated by Lys-155 to Gln-165, is highly sensitive to the chemical identity of the nucleotide situated in the binding pocket. This sensitivity to nucleotide identity is in sharp contrast to that observed for the "P-loop"-containing enzymes, in which the conformation of the binding motif is virtually unchanged in the presence or absence of nucleotides.

The biological importance of proteins that bind and utilize ATP cannot be overstated. Nature has, indeed, evolved a variety of molecules to accommodate nucleotides such as ATP, and biochemists are now in the process of understanding these motifs on a detailed three-dimensional level. To date, the most commonly known and clearly the best-characterized ATP-bind-
during the formation of the formyl phosphate intermediate leads to a shift of the resulting Mg\(^{2+}\)-formyl phosphate toward the amine group of GAR. Asp-268 has been postulated to function as a general base to deprotonate the amine group of GAR, which then reacts with the carbonyl group of the acylphosphate intermediate (7). The binding site for formate is presently unknown.

Throughout the years, AMPPNP, first synthesized by Prof. Ralph Yount’s laboratory in 1971 (8), has gained widespread usage and acceptance as an adenosine nucleotide analog in biochemical studies. Other commonly employed adenosine nucleotide analogs include ADP/beryllium fluoride (9), ADP/aluminum fluoride (10, 11), AMPPCP, and ATP\(_{S}\). The ADP-aluminum fluoride complex is thought to specifically mimic the transition state for ATP hydrolysis (9–12). As with any substrate or nucleotide analog, however, some caution must be applied in interpreting the results from x-ray models. Here we describe and compare the high-resolution x-ray structures of PurT transformylase complexed with Mg\(^{2+}\)ATP/GAR, Mg\(^{2+}\)ATP, Mg\(^{2+}\)AMPPCP, Mg\(^{2+}\)ATP\(_{S}\), or Mg\(^{2+}\)ADP to address the subtle changes in protein structure that occur upon adenosine nucleotide analog binding. PurT transformylase was chosen as the subject for this investigation because crystals of the enzyme diffract to at least 1.75 Å resolution and, in one case, to 1.05 Å resolution. The studies presented here demonstrate that the conformations of the polypeptide chain between Lys-155 to Gln-165 and Gln-225 to Tyr-230 in PurT transformylase are dependent upon the identity of the nucleotide analog located within the active site. Because this research represents the first systematic analysis of nucleotide analog binding within the ATP-grasp fold, the concepts gleaned from this study may be directly applicable to other members of the enzyme superfamily.

**EXPERIMENTAL PROCEDURES**

**Purification and Crystallization of PurT Transformylase**—Recombinant PurT transformylase was expressed in an *E. coli* overproducing strain BL21(DE3), pLysS, carrying the PurT gene on a pET-22b plasmid and purified as described previously (7).

Crystals of the Mg\(^{2+}\)ATP, Mg\(^{2+}\)ATP\(_{S}\), and Mg\(^{2+}\)AMPPCP complexes were grown by macroseeding into batch setups at 4 °C. The protein stock solutions, at 22 mg/ml were adjusted with ATP (or ATP\(_{S}\) or AMPPCP), MgCl\(_{2}\), and NaCl such that the final concentrations were

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**SCHEME 1.** PurT transformylase functions in purine biosynthesis by catalyzing the formylation of glycinamide ribonucleotide or GAR.

**FIG. 1.** Ribbon representation of one subunit of PurT transformylase. All figures were prepared with the program MOLSCRIPT (22) (BobScript, version 2.01, copyright Robert Esnouf, 1994–1996). X-ray coordinates utilized for this figure were determined by this laboratory and can be obtained from the Protein Data Bank (1EZ1). Bound AMPPNP and GAR are displayed in ball-and-stick representations. The A-, B-, and C-domains, defined by Thr-2 to Ala-122, Glu-123 to Gly-196, and Val-197 to Gly-392, are color-coded in yellow, green, and blue, respectively.

**FIG. 2.** Close-up view of the active site of PurT transformylase. The AMPPNP and GAR moieties are highlighted in yellow and pink bonds, respectively. Those amino acids located within ~3.2 Å of the AMPPNP and GAR moieties are shown in stereo. The amino group of GAR that is formylated during the reaction mechanism is indicated by the asterisk.
X-ray Structure of Glycinamide Ribonucleotide Transformylase

### Table I

| Complex   | Resolution | Independent reflections | Completeness | Redundancy | Avg I/Avg or(I) | \(R_{sym} \) |
|-----------|------------|-------------------------|--------------|------------|-----------------|--------------|
| ATP/GAR   | 30.0–1.60  | 108,032                 | 97.0         | 3.1        | 18.9            | 4.4          |
| ATP       | 1.67–1.60* | 12,764                  | 92.0         | 2.0        | 3.4             | 21.2         |
| AMPPPCP   | 30.0–1.60  | 106,671                 | 95.0         | 2.3        | 17.6            | 5.4          |
| ATP*S     | 30.0–1.75  | 82,695                  | 96.0         | 2.5        | 18.2            | 6.0          |
| ADP       | 1.67–1.75  | 9407                    | 85.0         | 1.4        | 4.4             | 21.5         |
| ADP       | 30.0–1.05  | 365,075                 | 93.0         | 3.6        | 22.2            | 6.6          |
| ADP       | 1.09–1.05  | 29,988                  | 75.5         | 2.5        | 1.9             | 31.4         |

\( \text{R}_{sym} = \left( \sum |F_o| - |F_c| / \sum |F_o| \right) \times 100. \)

*Statistics for the highest resolution bin.

### Table II

#### Relevant least-squares refinement statistics

| Complex   | ATP/GAR | ATP | AMPPPCP | ATP*S | ADP |
|-----------|---------|-----|---------|-------|-----|
| Resolution limits (Å) | 30.0–1.60 | 30.0–1.60 | 30.0–1.60 | 30.0–1.75 | 30.0–1.05 |
| \(\text{R}_{\text{f}} \) (overall) (%/no. of reflns) | 18.2/108,032 | 18.0/106,671 | 18.4/105,396 | 18.7/82,695 | 19.3/86,675 |
| \(\text{R}_{\text{f}} \) (working) (%/no. of reflns) | 18.1/97,230 | 18.9/94,855 | 18.4/74,425 | 18.9/62,570 | 18.8/62,570 |
| \(\text{R}_{\text{f}} \) (free) (%/no. of reflns) | 22.8/10,802 | 23.0/10,671 | 22.3/10,541 | 23.3/8,270 | 21.4/36,505 |
| No. of protein atoms | 5917b | 5988c | 5932d | 5989c | 6033e |
| No. of hetero-atoms | 979b | 1101c | 1031d | 755c | 1175e |

\* These include multiple conformations for Leu-3, Arg-43, Ser-56, Arg-252, Ile-276, and Ser-361 in Subunit I and Cys-271, Arg-283, and Gln-349 in Subunit II.

\* These include multiple conformations for Cys-271 in Subunit I and Cys-271 in Subunit II.

\* These include multiple conformations for Ile-39 in Subunit I and Cys-271 in Subunit II.

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### X-ray Data Collection and Processing

All x-ray data sets were collected using a HiStar (Bruker AXS) area detector system using CuKα radiation generated from a Rigaku RU200 rotating anode generator operated at 50 kV and 90 mA and equipped with Göbel focusing optics. The x-ray data sets were processed with SAINT (Bruker AXS, Inc.) and internally scaled with XSCALIBURE. X-ray data from the crystals of the PurT transformylase-Mg\(^{2+}\) ADP complex to 1.65 Å resolution were collected at Argonne Advanced Photon Source, Structural Biology Center line 19-ID. These data were processed with HKL2000 and scaled with SCALEPACK (13). Relevant intensity statistics are presented in Table I.

All of the structures reported here were solved by Difference Fourier analyses using the PurT transformylase-Mg\(^{2+}\) AMPPPNP complex coordinates as the starting models (7). Alternate cycles of least-squares refinements with the software package TNT (14) and manual inspections of the models reduced the conventional \(R\)-factors to \(\leq 19\%\) for all measured x-ray data. Relevant refinement statistics can be found in Table II.

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5, 10, and 250 mM, respectively, and the protein concentrations were 20 mg/mL. For each enzyme:nucleotide complex crystallization trial, the protein solution was mixed 1:1 with precipitating solutions containing 16–24% (w/v) methylenephosphoryl polyethylene glycol (MPEG) 5000, 100 mM MOPS (pH 6.7), and 100 mM MgCl\(_2\). The resulting solutions were spun in a microfuge to clarify them, and 25-μl aliquots were placed onto Plexiglas depression trays. A single crystalline seed was introduced into each drop, which was then subsequently seeded with a overslip to prevent evaporation. Crystals with overall dimensions of 1.0 × 0.6 × 0.5 mm were obtained within 2–3 weeks. The crystals belonged to the space group P2\(_1\)2\(_1\)2 with typical unit cell dimensions of \(a = 62.3\) Å, \(b = 179.5\) Å, and \(c = 75.7\) Å and one dimer per asymmetric unit. Each subunit contains 392 amino acid residues. Preparation and crystallization of the protein complex with ADP were similar to those of the ATP analogs.

For preparation of the PurT transformylase ternary complex with Mg\(^{2+}\) ATP and GAR, crystals were first grown in the presence of Mg\(^{2+}\) ATP and subsequently transferred to a solution containing 20% (w/v) methylenephosphoryl polyethylene glycol (MPEG) 5000, 50 mM MgCl\(_2\), 500 mM NaCl, 2.5 mM ATP, 100 mM MOPS (pH 6.7), and 1 mM GAR and allowed to soak for ~12 h.

**X-ray Data Collection and Processing**—All x-ray data sets were collected on flash-frozen crystals that had been transferred to cryoprotectant solutions containing 2.5 mM ATP (or other relevant nucleotide analogs), 500 mM NaCl, 25% (w/v) methylenephosphoryl polyethylene glycol (MPEG) 5000, 50 mM MgCl\(_2\), 100 mM MOPS (pH 6.7), and 15% (v/v) ethylene glycol. With the exception of the enzyme:Mg\(^{2+}\) ADP complex, all x-ray data sets were collected with a HiStar (Bruker AXS) area detector system using CuKα radiation generated from a Rigaku RU200 rotating anode generator operated at 50 kV and 90 mA and equipped with Göbel focusing optics. The x-ray data sets were processed with SAINT (Bruker AXS, Inc.) and internally scaled with XSCALIBURE. X-ray data from the crystals of the PurT transformylase-Mg\(^{2+}\) ADP complex to 1.65 Å resolution were collected at Argonne Advanced Photon Source, Structural Biology Center line 19-ID. These data were processed with HKL2000 and scaled with SCALEPACK (13). Relevant intensity statistics are presented in Table I.

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G. Wesenberg and I. Rayment, unpublished results.
Quality of the X-ray Models

The quality of the models, as judged from Table II, is excellent with regard to both conventional R-factors and overall geometrical constraints. Ramachandran plots for all of the models reveal that 95% of the non-glycinyl residues fall well within the allowed regions. In each model, the only significant outlier in the Ramachandran plot is Leu-8. The electron density for this residue is unambiguous. The quality of the electron density for the solvent is especially revealing. Fig. 3 shows the observed electron density corresponding to a bound MOPS buffer molecule. This molecule is positioned within the subunit:subunit interface of the physiological dimer. The morpholino ring of the buffer is located in a pocket that includes the side chain of Leu-8, whereas the sulfonic acid moiety forms a hydrogen bond with the peptidic NH group of Ser-56. In all of the structures reported here, the electron density corresponding to Subunit I in the asymmetric unit was somewhat better ordered than that for Subunit II. Hence the following discussions refer only to Subunit I of the dimer.

RESULTS AND DISCUSSION

The work described here unequivocally demonstrates that in the ATP-grasp proteins, the conformation of the binding loop, which in this study is referred to as the “T-loop,” is highly dependent upon both the presence or absence of nucleotide and the nature of the moiety occupying the γ-phosphate position. This is in sharp contrast to the P-loop enzymes, in which the conformation of the nucleotide binding motif is essentially insensitive to the presence or absence of ligands. Also, unlike that observed for the P-loop motif, there is not a characteristic signature sequence in the primary structure of the T-loops. In all cases, however, the T-loop begins with either a lysine residue, as in PurT transformylase (7), glutathione synthetase (15), D:alanine D:alanine ligase (16), biotin carboxylase (17), and N5-carboxylaminoimidazole ribonucleotide synthetase (18), or an arginine residue, as in carbamoyl phosphate synthetase (19, 20). These residues participate in electrostatic interactions with the γ-phosphates of the adenosine nucleotide ligands. Superpositions of the T-loops in PurT transformylase, carbamoyl phosphate synthetase (19), glutathione synthetase (15), and D:alanine D:alanine ligase (16) are displayed in Fig. 4. The T-loops, which connect the second and third β-strands of the B-domains, typically contain either Type I’ or Type III’ turns that participate in hydrogen bonding interactions with the β- and γ-phosphoryl oxygens. As can be seen in the case of the PurT transformylase-Mg2+ADP complex, residues Ser-160...
to Gly-162 are disordered but adopt a Type I’ turn (Ser-160 to Lys-163) conformation upon Mg$^{2+}$ ATP binding.

The first structure of PurT transformylase to be solved was that of the enzyme complexed with Mg$^{2+}$ AMPPNP and GAR (7). For the sake of subsequent comparisons, a close-up view of the electron density corresponding to the AMPPNP moiety (red) and a portion of the T-loop (blue). The electron densities for the two observed magnesium ions are displayed in green. For the figure, the electron density map was calculated with coefficients of the form (2$F_o$ – $F_c$), where $F_o$ was the native structure factor amplitude, and $F_c$ was the calculated structure factor amplitude. The map was contoured at 1σ. Possible hydrogen bonding interactions (within 3.2 Å) between the protein and the purine nucleotide are indicated by the dashed lines in b.

FIG. 5. Structure of PurT transformylase complexed with Mg$^{2+}$ AMPPNP and GAR. Shown in a is a portion of the electron density map corresponding to the AMPPNP moiety (red) and a portion of the T-loop (blue). The electron densities for the two observed magnesium ions are displayed in green. For the figure, the electron density map was calculated with coefficients of the form (2$F_o$ – $F_c$), where $F_o$ was the native structure factor amplitude, and $F_c$ was the calculated structure factor amplitude. The map was contoured at 1σ. Possible hydrogen bonding interactions (within 3.2 Å) between the protein and the purine nucleotide are indicated by the dashed lines in b.

The adenine ring of the nucleotide is anchored to the protein via electrostatic interactions with the backbone carbonyl oxygen of Gly-196 and with the peptidic NH group of Val-198, a feature conserved in members of the ATP-grasp superfamily with known three-dimensional structures. In addition to these backbone atoms, O$^\gamma$ of Glu-195 also interacts with the adenine ring of the nucleotide. In some cases, such as carbamoyl phosphate synthetase or glutathione synthetase, this glutamate is replaced with an aspartate or glutamine, respectively. Both Lys-155 and Arg-114 provide key electrostatic interactions with the α- and β-phosphoryl oxygens, respectively. The position occupied by Arg-114 in PurT transformylase is typically an arginine or lysine residue in other members of the ATP-grasp superfamily. The two ob-
served Mg$^{2+}$ ions are octahedrally coordinated and bridged together by the carboxylate side chain of Glu-279, which is strictly conserved among ATP-grasp proteins of known structure. Glu-267 in PurT transformylase serves as a bidentate ligand to one of the Mg$^{2+}$ ions. In some members of the ATP-grasp superfamily, this residue is replaced with a glutamine (as in carbamoyl phosphate synthetase (19)) or an aspartate (as in glutathione synthetase or D:alanine D:alanine ligase (15, 16)). Glu-203 in PurT transformylase hydrogen bonds to both the 2'- and 3'-hydroxyl groups of the nucleotide ribose, which adopts the C$_{3'}$-endo conformation. This 2'- and 3'-hydroxyl-bridging glutamate is conserved in most of the ATP-grasp family members including carbamoyl phosphate synthetase, where it also functions as a ligand to the bound potassium ion (19). Interestingly, in glutathione synthetase or biotin carboxylase, this glutamate is replaced with an aspartate or a histidine, respectively (15, 17). In PurT transformylase, a water molecule lies within hydrogen bonding distance to the nitrogen bridging the $\beta$- and $\gamma$-phosphates of the AMPPNP.

It is widely accepted that Mg$^{2+}$ AMPPNP serves as an excellent mimic of Mg$^{2+}$ ATP (8). Fig. 6a shows the electron density corresponding to the Mg$^{2+}$ ATP and the T-loop in the Mg$^{2+}$ ATP-GAR complex of PurT transformylase. A cartoon of the hydrogen bonding pattern between the protein and Mg$^{2+}$ ATP is given in Fig. 6b. Although the electron density for Ser-160 is weak, it is clear, by comparing Figs. 5a and 6a, that
the side chain of Ser-160 has moved out of the binding pocket such that it no longer forms a hydrogen bond with a γ-phosphoryl oxygen of the nucleotide. A second major difference between these two adenosine nucleotide-GAR complexes of PurT transformylase occurs at Gln-165. In the Mg$^{2+}$ ATP-GAR protein complex, the carboxamide nitrogen of Gln-165 lies at 2.9 Å from the bridging $\alpha,\beta$-phosphorus oxygen. This corresponding distance in the Mg$^{2+}$ AMPPNP-GAR-protein complex is 3.6 Å. In addition to the changes in side chain conformations for Ser-160 and Gln-165, the loop delineated by Gln-225 to Tyr-230 also varies depending upon the nature of the nucleotide present in the active site. A distance of 5.5 Å is observed between the $\alpha$-carbon of Gly-228 and C-5 of the AMPPNP ribose. Upon Mg$^{2+}$ ATP binding, however, the loop closes down such that the observed distance is 4.2 Å.

Strikingly, the electron density corresponding to Ser-160 and Ser-161 in the PurT transformylase Mg$^{2+}$ ATP complex is very well ordered, as seen in Fig. 7a. In the absence of the GAR substrate, a third magnesium ion is observed in the active site. As indicated in the hydrogen bonding cartoon in Fig. 7b, this magnesium ion is coordinated by the carboxylate side chain of...
Asp-286, one of the γ-phosphorly oxygens of the nucleotide, and four water molecules. Note that in the complexes of PurT transformylase with GAR, the carboxylate side chain of Asp-286 lies within hydrogen bonding distance of the amino group of GAR, and it is this residue that is thought to function as the active site base in the reaction mechanism (7).

As indicated in Fig. 7b, an ethylene glycol molecule is coordinated to the magnesium ion that bridges the β- and γ-phosphates of the ATP. This position is occupied by a water molecule in all of the other structures presented here.

The next structure to be solved in this investigation was that of PurT transformylase complexed with Mg²⁺AMPPCP. As can be seen from the electron density in Fig. 8a, replacement of the bridging β,γ-oxygen with a methylene group resulted in a disordering of the T-loop. The third magnesium site observed in the PurT transformylase-Mg²⁺ATP structure is not present. Other than this change, however, the overall hydrogen bonding pattern exhibited between the Mg²⁺AMPPCP ligand and the protein is similar to that observed for the Mg²⁺ATP-protein complex.

In myosin subfragment-1, as in other P-loop enzymes, the binding of Mg²⁺ATPγS results in little perturbation of the protein structure (21). When complexed with PurT transformylase, however, this sulfur-containing nucleotide analog, like Mg²⁺AMPPCP, disrupts the conformation of the T-loop, as can be seen in Fig. 8b. Note that the sulfur group of the nucleotide was modeled into the electron density map as indicated in Fig. 8b in light of the larger lobe of electron density observed in the map at this position and also taking into account the general ligation preference of magnesium ions for oxygen rather than sulfur atoms. The difference in the quality of the electron density for the T-loop with bound Mg²⁺ATP (Fig. 7a) versus Mg²⁺ATPγS (Fig. 8b) strikingly emphasizes that in the ATP-grasp enzymes, the chemical nature of the terminal phosphate

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**Fig. 8.** Electron densities for the T-loops with either Mg²⁺AMPPCP or Mg²⁺ATPγS bound in the active site of PurT transformylase. The observed electron densities for the T-loops with either Mg²⁺AMPPCP or Mg²⁺ATPγS bound in the active sites are displayed in a and b, respectively.
is critical for the polypeptide chain conformation, and, at least in the case of PurT transformylase, the Mg\(^{2+}\)-ATP\(^-\)/S is not an acceptable nucleotide analog.

The final structure solved in this study was that of the enzyme complexed with Mg\(^{2+}\)-ADP. Due to the high quality of the crystals, it was possible to determine the structure of this complex to a nominal resolution of 1.05 Å. Electron density corresponding to the nucleotide diphosphate is shown in Fig. 9a, and a cartoon of the hydrogen bonding pattern between the ligand and the protein is given in Fig. 9b. In this case, the electron density corresponding to the T-loop is completely disordered. The sole magnesium ion is coordinated by oxygen-containing ligands in a nearly exact octahedral ligation sphere. The ligand:metal:ligand bond angles range from 85.8° to 94.7°, with an average value of 90.0°. Bond distances between the magnesium ion and the \(-\) and \(-\)-phosphoryl oxygens are 1.8 Å, whereas the distances between the cation and the two waters are 1.7 Å and 1.9 Å, respectively. In the nucleotide triphosphate-protein complexes, the carboxylate side chain of Glu-267 functions as a bidentate ligand to the magnesium ion,
with each of its carboxylate oxygens lying with 2.1 Å of the magnesium ion. Upon Mg²⁺ADP binding in the active site, however, the side chain of Glu-267 swings away such that only O²⁻ of the carboxylate group ligates the cation (1.8 Å), whereas O¹⁻ is located at 3.2 Å from the magnesium ion. In addition to these changes, in the Mg²⁺ADP/protein model, N³⁺ of Gln-165 no longer participates in hydrogen bonds with the nucleotide. A superposition of the Mg²⁺ATP and Mg²⁺ADP complexes of PurT transformylase near the active site region is displayed in Fig. 10. In addition to the ordering of the T-loop upon nucleotide triphosphate binding, the polypeptide chain delineated by Gln-225 to Tyr-230 also adopts a different conformation such that the -carbon of Gly-228, for example, moves by ~3.0 Å. It can be speculated that the observed changes in polypeptide chain conformation may be required to facilitate both the binding of Mg²⁺ATP and the removal of Mg²⁺ADP after the reaction.

One of several key questions remaining with regard to the ATP-grasp enzymes is the chemical nature of the acylphosphate intermediate binding pocket. In the past, x-ray analyses of the binding modes for nucleotide analogs such as ADP/beryllium fluoride and ADP/aluminum fluoride in the P-loop enzymes have effectively addressed important structural questions regarding the catalytic mechanisms of these enzymes. With this in mind, intense efforts were put forth in this study on PurT transformylase to study the binding of ADP/beryllium fluoride and ADP/aluminum fluoride compounds to the enzyme in an effort to address the issue of the acylphosphate binding site. As stated previously, ADP/aluminum fluoride is thought to mimic the transition state of ATP hydrolysis and thus might help to identify the location of the acylphosphate intermediate in PurT transformylase. When the enzyme was crystallized in the presence of 5 mM Mg²⁺ADP/aluminum fluoride, however, only Mg²⁺ADP was observed in the active site. The same results occurred when the enzyme was crystallized in the presence of Mg²⁺ADP/beryllium fluoride or Mg²⁺ADP/vanadate. Interestingly, similar attempts to bind these ATP analogs to carbamoyl phosphate synthetase, another member of the ATP-grasp superfamily, also resulted in only Mg²⁺ADP being observed in the active site.³ In retrospect, the results with both PurT transformylase and carbamoyl phosphate synthetase are not surprising. As demonstrated here, the T-loop is exquisitely sensitive to the composition of the nucleotide and most likely is disordered in the presence of analogs such as Mg²⁺ADP/beryllium fluoride. Additional attempts to locate the formyl phosphate binding pocket in PurT transformylase have included crystallization experiments with phosphonoacetic acid or phosphonoformic acid (Scheme 2) and Mg²⁺ADP. Again, the resulting electron density maps clearly indicated the presence of only Mg²⁺ADP and GAR in the active site. Crystallization of PurT transformylase in the presence of ADP and acetyl phosphate (in the presence of either magnesium or manganese) resulted in the appearance of ATP in the active site, whereas crystallization of the protein in the presence of ADP/βS and acetyl phosphate resulted in only ADP/βS being observed in the active site.

One of the more interesting results from these high-resolution x-ray analyses of PurT transformylase is the difference in the side chain conformation of Ser-160 in the presence of Mg²⁺AMPPNP versus Mg²⁺ATP, both with bound GAR. In the former case, the side chain of Ser-160 swings in toward the γ-phosphate of the nucleotide and is located within 2.6 Å of a γ-phosphoryl oxygen. In the case of bound Mg²⁺ATP, however, the side chain of Ser-160 moves out toward the solvent such that Oγ is ~4.8 Å away from the same γ-phosphoryl oxygen. Given the relative orientation of Ser-160 in relationship to both the Mg²⁺ATP and the GAR (or Mg²⁺AMPPNP as indicated in Fig. 2), it might be speculated that it is involved in the positioning of formate. When the proper nucleotide, i.e. Mg²⁺ATP, is bound in the active site, the side chain of Ser-160 moves toward the solvent to allow formate to enter the binding region. Although there have been no site-directed mutagenesis analyses thus far targeting Ser-160, interestingly, when Gly-162 is changed to an isoleucine, there is a 317-fold reduction in kcat, with a 2-fold increase in Kₘ for ATP and GAR. More impor-

³ J. B. Thoden, F. M. Rauschel, and H. M. Holden, unpublished data.
tantly, however, the G162I mutant protein exhibited a 180-fold increase in $K_m$ for formate, suggesting a direct effect on the formate binding pocket (6). In addition, this mutation allowed the detection of formyl phosphate, indicating that in this mutant protein, the formyl phosphate was not held as tightly to the enzyme. Coupled with the x-ray structural data presented here, it seems likely that conformational changes in the T-loops of the ATP-grasp superfamily play critical roles in the formation and trapping of key reactive intermediates.

In light of these recent biochemical and x-ray crystallographic studies, site-directed mutagenesis experiments directed at Ser-160 are presently under way. An additional target for study is Asp-286. This residue has been postulated to function as the active site base for the deprotonation of the amine of GAR, which subsequently reacts with the carbonyl group of formyl phosphate. By changing this residue to an asparagine, for example, it may be possible to trap the reaction in the crystalline lattice at the stage of Mg$_2^+$ADP, formyl phosphate and GAR. Again, these experiments are presently under way.

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