Entrapment of 6-Thiophosphoryl-IMP in the Active Site of Crystalline Adenylosuccinate Synthetase from Escherichia coli*

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Crystal structures of adenylosuccinate synthetase from Escherichia coli complexed with \( \text{Mg}^{2+} \), 6-thiophosphoryl-IMP, GDP, and hadacidin at 298 and 100 K have been refined to \( R \)-factors of 0.171 and 0.206 against data to 2.8 and 2.5 Å resolution, respectively. Interactions of GDP, \( \text{Mg}^{2+} \) and hadacidin are similar to those observed for the same ligands in the complex of IMP, GDP, NO\(_3\), Mg\(^{2+}\) and hadacidin (Poland, B. W., Fromm, H. J. & Honzatko, R. B. (1996). J. Mol. Biol. 264, 1013–1027). Although crystals were grown from solutions containing 6-mercapto-IMP and GTP, the electron density at the active site is consistent with 6-thiophosphoryl-IMP and GDP. Asp-13 and Gln-224 probably work in concert to stabilize the 6-thioanion of 6-mercapto-IMP, which in turn is the nucleophile in the displacement of GDP from the γ-phosphate of GTP. Once formed, 6-thiophosphoryl-IMP is stable in the active site of the enzyme under the conditions of the structural investigation. The direct observation of 6-thiophosphoryl-IMP in the active site is consistent with the putative generation of 6-phosphoryl-IMP along the reaction pathway of the synthetase.

6-Mercapto-purine is used in the treatment of pediatric leukemia and other cancers (1). The drug is transformed enzymically to 6-mercapto-IMP and then into other derivatives such as 6-thio-GTP, 6-thio-ITP and 6-thiophosphoryl-IMP. 6-Thio-GTP can be incorporated into DNA, increasing the susceptibility of DNA to damage. 6-Thiophosphoryl-IMP is a potent inhibitor of phosphoribosylpyrophosphate amidotransferase, suppressing \( \text{de novo} \) purine biosynthesis, and 6-mercapto-ITP is a potent inhibitor of RNA polymerase. Depletion of the adenine nucleotide pool (specifically ATP) by the action of 6-thiophosphoryl-IMP reduces levels of S-adenosyl-L-methionine, which in turn impedes methylation of DNA and RNA. Although mechanisms for the cytotoxic effects of 6-mercapto-IMP are known, the metabolic pathways by which 6-mercapto-IMP is inactivated as a drug are unclear.

Adenylosuccinate synthetase (EC 6.3.4.4) is likely to play a role in the metabolic effects of 6-mercapto-IMP. The synthetase governs the first committed step in the \( \text{de novo} \) biosynthesis of AMP (2), GTP + IMP + L-aspartate ⇄ GDP + \( \text{P} + \) adenylosuccinate. 6-Mercapto-IMP is a competitive inhibitor with respect to IMP (3–5) and likely inhibits the enzyme \( \text{in vivo} \). The combination of adenylosuccinate synthetase with adenylosuccinate, GDP, and thiophosphate (reverse reaction) leads to the slow generation of 6-mercapto-IMP, GTP, and aspartate (6). However, 6-mercapto-IMP, GTP, and aspartate in the presence of enzyme (forward reaction) gives no detectable level of adenylosuccinate. This apparent violation of microscopic reversibility has been explained by an equilibrium constant that significantly favors GTP, 6-mercapto-IMP, and aspartate over GDP, adenylosuccinate, and thiophosphate (6). The interaction of 6-mercapto-IMP with adenylosuccinate then may contribute to the suppression of \( \text{de novo} \) purine biosynthesis but only if \( \text{in vivo} \) levels of IMP are low relative to those of 6-mercapto-IMP.

We report here refined crystal structures at 298 and 100 K of adenylosuccinate synthetase from \( \text{Escherichia coli} \), grown in the presence of 6-mercapto-IMP, GTP, \( \text{Mg}^{2+} \), and hadacidin. Hadacidin (see Fig. 1), a fermentation product of \( \text{Penicillium frequentans} \) (7), is a competitive inhibitor (\( K_i \sim 10^{-6} \text{M} \)) with respect to aspartate (4–5); the only known function of hadacidin is the inhibition of adenylosuccinate synthetase (8). In the mechanism proposed for the synthetase by Lieberman (9), the γ-phosphate of GTP is transferred to the 6-oxygen of IMP, whereafter aspartate displaces phosphate from the 6-phosphoreryl intermediate to form adenylosuccinate. Although the Lieberman mechanism enjoys substantial support from the literature (10–12), no study has provided direct evidence for the formation of a 6-phosphoryl intermediate. Furthermore, 6-phosphoryl-IMP has never been isolated or synthesized. Regardless of temperature, however, a 6-thiophosphoryl intermediate sits at the IMP binding site in the crystal structures, hence providing the first direct observation of 6-thiophosphoryl-IMP and strong evidence in support of the catalytic mechanism proposed 40 years ago by Lieberman (9).

MATERIALS AND METHODS

Adenylosuccinate synthetase was prepared as described previously from a genetically engineered strain of \( \text{E. coli} \) (13–14). The protein migrates as a single band on SDS-polyacrylamide gel electrophoresis with an apparent relative molecular weight of 48,000. Hadacidin was provided by Drs. Fred Rudolph and Bruce Cooper (Dept. of Biochemistry and Cell Biology, Rice University). All other reagents came from Sigma.

Conditions for the growth of the 6-thiophosphoryl-IMP complex were adapted from Poland et al. (15), using the method of hanging drops. Droplets contained 2 μl of enzyme solution (50 mM imidazole, 75 mM succinate, 4 mM GTP, 4 mM 6-mercapto-IMP, 5 mM hadacidin, and 20 mg/ml protein (pH 6.5)) and 2 μl of a crystallization buffer (13% polyethylene glycol 8000 (w/w), 100 mM cacodylic acid/cacodylate (pH 6.5), and 200 mM magnesium acetate). The final pH of the crystallization buffer was 6.5. Wells contained 500 μl of the crystallization buffer. Crystals of approximately 0.5 mm in all dimensions and belonging to the space group \( \text{P}3_12_1 \) (\( a = b = 81.63 \) and \( c = 159.2 \) at 298 K, and \( a = b = 80.54 \) and \( c = 158.2 \) at 100 K) grew in about 1 week. The asymmetric unit consists of a monomer.

Data from the 6-thiophosphoryl-IMP complexes were collected on a...
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Siemens area detector at Iowa State University and were reduced by using XENGEN (16). Data sets were 98% complete (see Table I).

Starting phases for the 6-thiophosphoryl-IMP complex were calculated from the IMP/NO$_3^-$ complex (15), omitting coordinates for water molecules, NO$_3^-$, and IMP. Refinement of the structures involved manual fitting of models to the electron density, using a Silicon Graphics 4D-25 and the program TOM (17), followed by a cycle of refinement using XPLOR (18) on a Silicon Graphics 4D-35. Constants of force and geometry for the protein came from Engh & Huber (19). The geometry of hadacidin was based on a related structural fragment (20), retrieved by a search of the Cambridge Data Base. Models of hadacidin with a planar nitrogen and with tetrahedral nitrogens in L- and D-configurations were refined individually. In early rounds of refinement, both crystal structures were heated to 2000 K and then cooled in steps of 25–300 K. In later rounds of refinement, the systems were heated to 1000 or 1500 K but then cooled in steps of 10 K. After the slow cooling protocol was completed (at 300 K), the models were subjected to 120 steps of conjugate gradient minimization, followed by 20 steps of individual B-parameter refinement. Individual B-parameters were subject to the following restraints: nearest neighbor, main chain atoms, 1.5 Å$^2$; next to nearest neighbor, main chain atoms, 2.0 Å$^2$; nearest neighbor, side chain atoms, 2.0 Å$^2$; and next to nearest neighbor, side chain atoms, 2.5 Å$^2$.

Water molecules were added if (i) electron density at a level of 2.5$\sigma$ was present in maps based on Fourier coefficients (F(iii) - F(obs))$^{\text{calc}}$, and (ii) acceptable hydrogen bonds could be made to an existing atom of the model. If after refinement a site for a water molecule fell beyond 3.3 Å from its nearest neighbor, that site was omitted from the model. In addition, water molecules were deleted from the model if their thermal parameters exceeded 80 Å$^2$. Harmonic restraints (50 kcal/mol) were placed on the positions of oxygen atoms of water molecules to allow new water molecules to relax by adjustments in orientation. Occupancies of water molecules were not refined due to the high correlation between occupancy and thermal parameters for data of 2.6 Å nominal resolution. Thus solvent sites with B values between 50 and 80 Å$^2$ probably represent water molecules with occupancy parameters below 1.0 and thermal parameters substantially lower than those reported from the refinement.

RESULTS

Quality of the Refined Models—The model reported here has been deposited with the Protein Data bank, Brookhaven National Laboratory (code 1NHT). The method of Luzzati (21) indicates an uncertainty in coordinates of 0.30 Å. The amino acid sequence used in refinement is identical to that reported by Silva et al. (14). Results of the refinement are in Table I.

The Ramachandran plot (22) for the structures reported here are comparable with those of Poland et al. (15). As in other crystal forms of the synthetase (14–15, 23–25), the most apparent outlier is Gln-10, which exists in the same conformation in each of the five independent polypeptide chains of three crystal forms. The program PROCHECK (26) indicates better stereochemistry for both the low and room temperature models. The program is a pictorial summary of the important interactions of ligands in the active site of the synthetase.

We focus, then, on the one feature that sets the present work apart from the former studies. The IMP and NO$_3^-$ sites are connected by continuous electron density, which we can interpret only as a molecule of 6-thiophosphoryl-IMP (Fig. 4). Omit maps are in complete agreement with electron density maps generated from coefficients $2F_{\text{obs}} - F_{\text{calc}}$. Thermal parameters of atoms of 6-thiophosphoryl-IMP are comparable with those of the IMP/NO$_3^-$ complex (15).

Ligand Binding Sites—The complex of 6-thiophosphoryl-IMP, GDP, Mg$^{2+}$, and hadacidin (Fig. 1) is isoformous to that of IMP, GDP, NO$_3^-$, Mg$^{2+}$, and hadacidin (15) and to that of hydantocidin 5'-phosphate, GDP, HPO$_4^{2-}$, Mg$^{2+}$, and hadacidin (25). A view of one monomer of the synthetase dimer with associated ligands appears in Fig. 2. Conformational changes in the synthetase upon ligation are described by Poland et al. (15) as are the interactions of GDP, Mg$^{2+}$, and hadacidin, which are the same here within experimental error. Fig. 3 is a pictorial summary of the important interactions of ligands in the active site of the synthetase.

TABLE I
Refinement statistics for adenylosuccinate synthetase

<table>
<thead>
<tr>
<th>Resolution limit (Å)</th>
<th>P3$_{21}$ 298 K</th>
<th>P3$_{21}$ 100 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of measurements</td>
<td>156,061</td>
<td>161,473</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>16,429</td>
<td>22,201</td>
</tr>
<tr>
<td>Completeness of data set (%)</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>Completeness of data in the last resolution shell (%)</td>
<td>95 (2.90–2.80Å)</td>
<td>96 (2.61–2.50 Å)</td>
</tr>
<tr>
<td>R$_{merge}$</td>
<td>9.7</td>
<td>7.9</td>
</tr>
<tr>
<td>Number of reflections in refinement$^b$</td>
<td>12,221</td>
<td>19,932</td>
</tr>
<tr>
<td>Number of solvent sites</td>
<td>4,622</td>
<td>5,516</td>
</tr>
<tr>
<td>Number of atoms$^c$</td>
<td>156</td>
<td>404</td>
</tr>
<tr>
<td>R-factor$^d$</td>
<td>0.171</td>
<td>0.206</td>
</tr>
<tr>
<td>R-free$^d$</td>
<td>0.250</td>
<td>0.255</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>5 to 2.8</td>
<td>5 to 2.5</td>
</tr>
<tr>
<td>Mean B (Å$^2$) for protein</td>
<td>26</td>
<td>17</td>
</tr>
<tr>
<td>Root mean square deviations</td>
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<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.016</td>
<td>0.013</td>
</tr>
<tr>
<td>Bond angles (deg)</td>
<td>2.01</td>
<td>1.89</td>
</tr>
<tr>
<td>Dihedral angles</td>
<td>24.7</td>
<td>24.8</td>
</tr>
<tr>
<td>Improper dihedral angles (deg)</td>
<td>1.61</td>
<td>1.63</td>
</tr>
</tbody>
</table>

$^a$ R$_{merge} = \Sigma_i \Sigma_j |I_i| - (I_i)\Sigma_i |I_i|$, where $i$ runs over multiple observations of the same intensity and $j$ runs over all crystallographically unique intensities.

$^b$ All data in the resolution ranges indicated.

$^c$ Includes hydrogens linked to polar atoms.

$^d$ R-factor based on 10% of the data: the data randomly cull and not used in the refinement.

FIG. 1. Diagram of hadacidin and of 6-thiophosphoryl-IMP, showing labels of atoms.
the surrounding protein, suggesting full occupancy of the ligand. Thus, the electron density between the 6-sulfur and the phosphoryl group cannot arise as a consequence of the mutually exclusive binding of phosphate and 6-mercapto-IMP, with each ligand in partial occupancy. Finally, a model for 6-thiophosphoryl-IMP fits the electron density well, leaving behind no significant density in difference maps. Indeed, a difference map, based on observed data from the IMP/NO$_3$ complex and calculated phase angles from the IMP/NO$_3$ complex, reveals a strong and well defined peak of electron density in the area between the 6-oxygen of IMP and the nitrogen of NO$_3$ (Fig. 4).

Interactions involving the 5'-phosphate, the ribose, and the base (exclusive of the 6-thiophosphoryl group) of 6-thiophosphoryl-IMP are the same as those of IMP in an earlier study (15). The 5'-phosphate hydrogen bonds with OG1 of Thr-129, backbone amide 129, ND2 of Asn-38, OG1 of Thr-239, and the guanidinium group of Arg-143 of the symmetry-related monomer (Table II). In addition, three water molecules, 554, 636, and 732, mediate interactions between the 5'-phosphate and the protein. The 5'-phosphate of 6-thiophosphoryl-IMP along with the side chain of Asp-114 (conserved in all known sequences of the synthetase) lie at the N terminus of helix H4. No direct hydrogen bonds, however, exist between helix H4 and either Asp-114 or the 5'-phosphate.

The 2'-OH group of the ribose of 6-thiophosphoryl-IMP hydrogen bonds with the guanidinium of Arg-303 and the 3'-OH hydrogen bonds with waters 524 and 699, which in turn interact with backbone carbonyls 126 and 273, respectively. Although the data are not of sufficient resolution to unambiguously determine the state of puckering of the ribose, the best fit to the electron density occurs with a low energy 2'-endo conformation (pseudorotation phase angle, 166°). The torsion angles O5'-C5'-C4'-C3' (γ by convention) and O4'-C1'-N9-C4 (χ by convention) are 44° (+synclinal) and −135° (anti), respectively.

The base of 6-thiophosphoryl-IMP interacts with the amide side chain of Gln-224 through its N7 and S6 atoms and with the side chain of Asp-13 through its N1 position. Interactions of 6-thiophosphoryl-IMP are dominated by the 6-thiophosphoryl group. Backbone amides 13, 40, and 224, NZ of Lys-16, NE2 of His-41, and NE2 of Gln-224 hydrogen bond with oxygen and sulfur atoms of the thiophosphoryl group (Fig. 5). In addition, one of the oxygens of the thiophosphoryl group coordinates to the Mg$^{2+}$. The oxygen atoms of the thiophosphoryl group correspond in position to the oxygens of NO$_3$ in the IMP/NO$_3$ complex.

![Fig. 2. Stereo view of bound ligands in relation to a trace of α-carbons of adenylosuccinate synthetase.](image1)

![Fig. 3. Diagram of selected hydrogen and coordinate bonding of ligands in the active site of adenylosuccinate synthetase.](image2)

![Fig. 4. Electron density associated with 6-thiophosphoryl-IMP from a 2F$_{obs}$ − F$_{calc}$ map (top). Difference map based on observed data from the 6-thiophosphoryl-IMP complex and the IMP/NO$_3$ complex (15), using calculated phases from the IMP/NO$_3$ complex (bottom). Contour level for both maps is 6-s, with a cover radius of 1.0 Å employed in the top illustration.](image3)
complex (15) and to three of four oxygens of HPO$_4^{2-}$ in the hydantocidin 5'-phosphate complex (25). His 41 interacts with the β-phosphate of GDP when NO$_3^-$ resides in the phosphoryl site, as observed in the IMP/NO$_3^-$ complex (15). In the hydantocidin 5'-phosphate complex (25), which has HPO$_4^{2-}$ at the phosphoryl site, His-41 interacts with the HPO$_4^{2-}$ molecule, similar to the interaction observed for His-41 here. Presumably the increase in formal electrostatic charge from −1 to −2 is responsible for the interaction of His-41 with the anion located in the phosphoryl site.

**DISCUSSION**

Markham and Reed (7) deduced a ΔG of +5 kcal/mol at pH 7 for the reaction: GTP + 6-thiophosphoryl-IMP + L-aspartate ⇌ GDP + thiophosphate + adenylosuccinate. Even in the absence of hadacidin, which leads to the dead end kinetic complex observed here, the combination of GTP, 6-mercapto-IMP, aspartate, Mg$^{2+}$, and enzyme should lead to undetectable levels of product. A plausible outcome of the crystallization of GTP, 6-mercapto-IMP, Mg$^{2+}$, and hadacidin, then, would be a 6-mercapto-IMP-GTP or 6-mercapto-IMP-GDP complex. The ratio of GDP to GTP in the active site would depend largely on the rate of hydrolysis of GTP in the bulk solvent and the rate of exchange of guanine nucleotides between the active site and the bulk solvent. Instead, the active site contains 6-thiophosphoryl-IMP and GDP. The presence of 6-thiophosphoryl-IMP in the active site indicates a lower free energy for the 6-thiophosphoryl-IMP/enzyme complex than for the substrate/enzyme complex. Thus, the enzyme must be a thermodynamic trap for the 6-thiophosphoryl intermediate.

Several factors favor the formation of 6-thiophosphoryl-IMP in the active site of the synthetase. First, the pK$_a$ of N1 of 6-mercapto-IMP is approximately 1–1.5 pH units lower than that of N1 of IMP. Thus, Asp-13, which is a putative catalytic base in the abstraction of the proton from N1 of IMP (15), may be more effective in stabilizing the ionized form of the 6-mercapto nucleotide relative to the 6-oxo nucleotide (Fig. 6). In addition, the S-P thioester bond (approximately 2.1 Å in length) is 0.5 Å longer than the O-P ester bond. The increased bond length allows the three oxygens of the 6-thiophosphoryl group to occupy the positions of oxygen atoms of NO$_3^-$ in the IMP/NO$_3^-$ complex (15). Markham and Reed (5) have shown synergy in the binding of IMP, GDP, Mg$^{2+}$, and NO$_3^-$ to the synthetase. The distance between N of NO$_3^-$ and the 6-oxygen of IMP in the IMP/NO$_3^-$ complex is 2.7 Å, implying a strong electrostatic interaction between an electron deficient N of NO$_3^-$ and an electron-rich 6-oxygen of IMP (15). In the present study, the 6-sulfur (presumably stabilized initially as a thioanion) and the phosphorus atom of PO$_3^-$ (putatively derived from the γ-phosphate of GTP and stabilized in the NO$_3^-$ site) form a covalent bond as a consequence of electrostatic interactions and the increased atomic radii of sulfur and phosphorus relative to oxygen and nitrogen.

The proposed mechanism for the formation of 6-thiophosphoryl-IMP in the active site appears in Fig. 6. The interaction involving Asp-13 identifies it as a possible catalytic base in the abstraction of the proton from N1 of 6-mercapto-IMP and generation of a 6-thioanion. In fact, the natural substrate IMP may be in the 6-oxoanion state, when bound to the active site (15). Gln-224 stabilizes the 6-thioanion further by a hydrogen bond interaction. Mutation of Asp-13 to alanine inactivates the synthetase completely (27), and mutation of Gln-224 to gluta-
nucleophilic displacement of thiophosphate from the 6-phosphoryl intermediate by the amino group of L-aspartate (Fig. 7).

The second reaction governed by the synthetase involves the bound GTP (Fig. 6). Both His-41 and Mg$^{2+}$ probably play important roles in stabilizing charge development on the β- and γ-phosphates of GTP in the transition state (15). A His-41 to asparagine mutant is inactive (27) and Mg$^{2+}$ is required for activity of the synthetase (9).

The 6-thioanion then displaces GDP from the γ-phosphate of bound GTP (Fig. 6). Both His-41 and Mg$^{2+}$ probably play important roles in stabilizing charge development on the β- and γ-phosphates of GTP in the transition state (15). A His-41 to asparagine mutant is inactive (27) and Mg$^{2+}$ is required for activity of the synthetase (9).

The second reaction governed by the synthetase involves the nucleophilic displacement of thiophosphate from the 6-phosphoryl intermediate by the amino group of L-aspartate (Fig. 7). The conformation and interactions of aspartate are inferred from enzyme-bound hadacidin (15). The putative conformation of bound aspartate favors a hydrogen bond between its β-carboxylate and its α-amino group, suggesting a catalytic function for the β-carboxylate in abstracting a proton from that amino group. No direct evidence is available regarding the catalytic role of the β-carboxylate of aspartate. However, an α-amino acid is a substrate of the synthetase only if it bears a negatively charged substituent with carboxylate-like geometry at the β-carbon (30). Hydroxylamine is the only known substrate for the synthetase that departs significantly from aspartate in structure and charge distribution. Hydroxylamine may not require the catalytic support of the β-carboxylate because the pK$_a$ of its amine is significantly lower than that of the α-amino group of aspartate.

Assuming a catalytic function for the β-carboxylate of aspartate (there are no other candidates provided by the enzyme (15)), a total of three hydrogen bonds, then, may be involved in promoting the second reaction: (i) the putative interaction between the α-amino and β-carboxylate groups of aspartate, (ii) the hydrogen bond between Asp-13 and N1 of the phosphoryl intermediate, and (iii) the hydrogen bond between His-41 and the 6-phosphoryl group. The first interaction enhances the nucleophilicity of the amino group, the second hydrogen bond enhances the electrophilicity of C6 of the intermediate, and the third hydrogen bond stabilizes the developing charge on the leaving group (thiophosphate in this case). The extent to which these three hydrogen bonds promote catalysis in the second reaction cannot be answered on the basis of the present study.

Poland et al. (15) suggest a more significant interaction between Asp-13 and Mg$^{2+}$ after formation of the phosphoryl intermediate, hence transforming Asp-13 from a catalytic base in the phosphotransfer step into a catalytic acid for the second reaction. In the present complex, we observe no significant movement of Asp-13 toward the Mg$^{2+}$.

Lieberman (9) was the first to propose the formation of 6-phosphoryl-IMP as an intermediate on the reaction pathway governed by the synthetase, but the results of his study are consistent with two other proposed mechanisms (2). Miller and Buchanan (31) suggest an attack of aspartate on C6 of IMP in concert with an attack by the 6-oxygen of IMP on the γ-phosphate of GTP. Markham and Reed (6) were unable to find spectral evidence for the formation of a 6-phosphoryl intermediate and, as a consequence, suggested that the addition of aspartate to C6 of IMP preceded the phosphorylation of O6.

Isotope exchange studies at equilibrium by Cooper et al. (12),

however, support the formation of 6-phosphoryl-IMP prior to the nucleophilic attack of aspartate. Furthermore, Bass et al. (11) demonstrated the chemical exchange of $^{18}$O from the $\beta,\gamma$-bridging position of GTP to the oxygens of the $\beta$-phosphate of GTP in the presence of enzyme and IMP or in the presence of enzyme, IMP, and succinate (as an analog of aspartate). Isotope exchange could occur only if the $\gamma$-phosphate dissociates from GTP for an interval long enough to allow the rotational isomerization of the $\beta$-phosphate group. The absence of labeled phosphate in solution demonstrated that the dissociated $\gamma$-phosphate remained bound to the enzyme. As IMP was required for isotope exchange, Bass et al. (11) suggested that the dissociated $\gamma$-phosphate existed as 6-phosphoryl-IMP. The observation here of 6-thiophosphoryl-IMP in the active site of the synthetase is entirely consistent with the results and conclusions of the isotope exchange studies.

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REFERENCES
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