Thiamine pyrophosphokinase transfers a pyrophosphate group from a nucleoside triphosphate, such as ATP, to the hydroxyl group of thiamine to produce thiamine pyrophosphate (TPP). Deficiencies in thiamine can result in the development of the neurological disorder Wernicke-Korsakoff Syndrome as well as the potentially fatal cardiovascular disease wet beriberi. Pyrithiamine is an inhibitor of thiamine metabolism that induces neurological symptoms similar to that of Wernicke-Korsakoff Syndrome in animals. However, the mechanism by which pyrithiamine interferes with cellular thiamine phosphoester homeostasis is not entirely clear. We used kinetic assays coupled with mass spectrometry of the reaction products and phosphoesters, thiamine monophosphate (TMP), and thiamine triphosphate (TTP), TPP is also the precursor for two other thiamine phosphates, thiamine monophosphate (TMP), and thiamine triphosphate (TTP) (4–6). The functions of TMP and TTP are less clear. TTP is found at low levels, but is present in bacteria, fungi, plants, invertebrates, and vertebrates. Lakaye et al. (7) showed that thiamine triphosphate is required for optimal growth in Escherichia coli during amine acid starvation and suggested that accumulation of TTP may be involved in a new signal pathway that helps the bacteria to adapt to stringent conditions. In humans, TTP has been found in peripheral nerve cells and has been proposed to be required for ion translocation in stimulated nerve cells. Nghiem et al. (8) showed that TTP is the phosphate donor for the phosphorylation of 43K rapsyn, which is a peripheral protein specifically associated with the nicotinic acetylcholine receptor (nAChR). The level of TTP seems to be highly regulated (9–11). The presence of TTP in organisms as diverse as prokaryotes through mammals suggests that it may play some fundamental role in cell metabolism and/or cell signaling.

Two thiamine analogs, oxythiamine and pyrithiamine, are known to induce symptoms associated with thiamine deficiency in both mice and rats (Fig. 1). However, the symptoms produced by the two analogs are distinct (12). Oxythiamine treatment produces anorexia and lethargy, but not the neurological deficits associated with Wernicke-Korsakoff Syndrome and oxythiamine does not appear to deplete thiamine levels (13). Oxythiamine is a competitive substrate of TPK and can be phosphorylated by TPK to form oxythiamine pyrophosphate (14). Oxythiamine pyrophosphate (OPP) binds to and inhibits TPK-dependent enzymes and induces a functional thiamine deficiency because the catalytically critical 4’-NH2 group of thiamine pyrophosphate is substituted by a hydroxyl group thereby rendering both the cofactor and enzyme non-functional. In contrast, pyrithiamine administration pro-

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The atomic coordinates and structure factors (code 2F17) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, IN University School of Medicine, 635 Barnhill Dr., Indianapolis, IN 46202-5122, Tel.: 317-278-2008; Fax: 317-274-4686; E-mail: thurley@iupui.edu.

2 The abbreviations used are: TPK, thiamine pyrophosphokinase; TPP, thiamine pyrophosphate; TMP, thiamine monophosphate; TTP, thiamine triphosphate; PPP, pyri-thiamine pyrophosphate; OPP, oxythiamine pyrophosphate; PDB, Protein Data Bank; RMSD, root mean squared deviation; HPPK, 6-hydroxy-methyl-7,8-dihydropterin pyrophosphokinase.

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Pyritihiamine as a Substrate for Thiamine Pyrophosphokinase*

Jing-Yuan Liu 1, David E. Timm 1, and Thomas D. Hurley 1 3

From the 4 Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5122 and 5 Eli Lilly and Co., Indianapolis, Indiana 46285
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FIGURE 1. The chemical structures of thiamine, oxythiamine, and pyrithiamine.

duces neurological symptoms and appears to deplete thiamine levels (15). The mechanism by which pyrithiamine inhibits TPK and whether it can be phosphorylated by TPK is still unclear (16). In addition, little is known about the mechanism by which TPK catalyzes its reaction and the role the enzyme plays in maintaining thiamine phosphoester homeostasis.

We have previously solved mouse TPK structures in complexes with thiamine (17) and TPP (18). In this report, we describe the ternary structure of mouse TPK complexed with pyrithiamine pyrophosphate (PPP) and AMP at 2.5-Å resolution. Our crystal structure as well as data generated by kinetic assays and mass spectrometry showed that pyrithiamine can be phosphorylated by TPK. Moreover, the identification of the nucleotide bound to mouse TPK provides additional insights into the catalytic mechanism of TPK.

EXPERIMENTAL PROCEDURES

Crystallization, Structure Solution, and Refinement—The recombinant His-tagged mouse TPK was purified by nickel-chelate chromatography and dialyzed and concentrated as described previously (17). Crystallization experiments were set up by incubating mouse TPK with pyrithiamine and MgATP prior to adding the equilibrium mixture of enzyme, substrate, and products to the previously determined crystallization conditions. The following protocol was used for these incubations: solutions containing 2.5 mM, 5.0 mM, 12.5 mM, or 25 mM of both, pyrithiamine and Mg$^{2+}$/ATP at pH 7.0 were mixed in a 1:2 volume ratio with an 11 mg/ml enzyme solution and incubated at room temperature for 45 min. These four separate mixtures created solutions that were 0.83 mM, 1.7 mM, 4.2 mM, and 8.3 mM in substrate concentration and 7.4 mg/ml (0.28 mg) in enzyme concentration. Based on the known kinetic properties of mouse TPK we estimated that 45 min was sufficient to reach equilibrium for these reaction mixtures. To initiate crystallization, 3 μl of the equilibrium reaction mixture was mixed with 2 μl of the crystallization solution containing 2.0 M ammonium sulfate, 0.1 M HEPES, and 3% polyethylene glycol 400, pH 7.1. The crystallization experiment utilized the hanging drop vapor diffusion geometry, and the trays were incubated at room temperature. Crystals appeared within 2 weeks. The size of the crystals obtained in this manner tends to be smaller at the higher concentrations of Mg$^{2+}$/ATP.

To make solutions for cryopreservation the crystals prior to exposure to x-rays, a second reaction was set up with 60 mM pyrithiamine, 120 mM MgATP, pH 7.0, and 0.15 μg/ml mouse TPK, and the reaction mixture was incubated at room temperature for 30 min. Then the reaction mixture was mixed with the mother liquor solution from which the crystal was obtained in a ratio of 1:2. Crystals were soaked with the mixture for another 30 min to 1 h before they were transferred to a similarly preincubated mixture to which 15% (v/v) glycerol had been added. The crystals were flash-frozen in a supercooled gaseous nitrogen stream operated at −180 °C and evaluated for diffraction quality. The diffraction data were collected to 2.5-Å resolution using the oscillation method and 0.5 degree oscillation widths at beam line 19ID at the Advanced Photon Source located at the Argonne National Laboratory. The raw diffraction data were indexed, integrated, and scaled using HKL2000 (19) and were converted into structure factors using the program package CCP4 (20). The isomorphous apo-mouse TPK structure, after removal of solvent molecules, was used directly to perform the first round of refinement. Models were manually adjusted using the program CORINA (23, 24). The parameter and topology file for AMP and PPP was generated by the program Xplo2D (20), with manual adjustments to the default weighting schemes for the dihedral angles.

Kinetic Studies—Kinetic activity assays were performed using a coupled enzyme system (26) that included mouse TPK, myokinase (Sigma), pyruvate kinase (Sigma), and lactate dehydrogenase (Sigma). In this assay, each mole of AMP generated by mouse TPK results in the oxidation of two moles of NADH. The disappearance of NADH was monitored at 340 nm. One unit is defined by the amount of enzyme required to convert 1 nmol of thiamine to TPP per minute. The 1-mM standard reaction mixture contains 0.1 M HEPES, pH 7.4, 40 mM phosphoenolpyruvate (Sigma), 6 mM MgCl$_2$, 5 mM ATP, pH 7.4, 400 μM pyrithiamine (Sigma), or thiamine (Sigma), 30 units of lactate dehydrogenase (Sigma), 30 units of pyruvate kinase (Sigma), 30 units of myokinase (Sigma), and 0.2 mM NADH (Sigma). The reaction was initiated by the addition of 50 μg of TPK.

To rule out the possibility that pyrophosphate was produced when pyrithiamine was the substrate, a reagent kit to detect pyrophosphate (Sigma) was used. This kit uses another coupled enzyme assay system consisting of fructose-6-phosphate kinase (pyrophosphate-dependent), aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase. The reaction was carried out by incubating a 0.5-mM reaction mixture containing 0.2 M HEPES, pH 7.4, 2 mM thiamine or pyrithiamine, 5 mM ATP, pH 7.4, and 5 mM MgCl$_2$. The reactions were stopped by heating at 100 °C for 5 min, at which point 17 μl of the reaction mixture were removed and mixed with 983 μl of the pyrophosphate reagent kit containing NADH. The reaction was monitored spectrophotometrically at 340 nm for the oxidation of NADH.

Mass Spectroscopy Analysis—We attempted to detect the existence of PPP in the reaction mixture directly by electrospray ionization mass spectrometry. A 0.5-mM reaction mixture containing 100 μM MgCl$_2$, 500 μM ATP, pH 7.4, 15 μg of mouse TPK, and 1 mM pyrithiamine or 1 mM thiamine was incubated for 30 min at 37 °C. A negative control reaction was also prepared without added enzyme. After 30 min of incubation, methanol was mixed with reaction mixture to achieve a final concentration of 50% (v/v). The assay sample was injected into a Finnigan LCQ DecaMS$^2$ system at a rate of 3.00 μl/min. Formic acid was added to a final concentration of 0.1% to samples analyzed in positive ion mode, whereas 1% ammonium hydroxide was added to samples analyzed in the negative ion mode. Positive and negative ions were scanned by the Xcalibur software package that fell between $m/z$ ranges of 100 and 1000. The spray voltage was set at 4.00 kV. Data from 10 continuous scans were collected and saved.

RESULTS

Structure of Mouse TPK Complexed with Pyrithiamine Pyrophosphate and MgAMP—Purified mouse TPK was incubated with pyrithiamine and Mg$^{2+}$/ATP to generate an equilibrium substrate/product
The AMP molecule binds to the main chain oxygen of Gly129 and the N1 atom. The location of these subunits and adopts the F-conformation (Fig. 2). Similar to TPP in the TPP-complexed structure of mouse TPK, PPP is found at the interface between the two subunits of the dimeric enzyme. However, subsequent refinement and difference feature analysis suggests that only the A subunit contains a fully occupied AMP molecule, whereas the B subunit appears to contain mixed occupancy of AMP and the buffer molecule HEPES. The latter of which is modeled into the B subunit as the resulting difference maps show fewer significant positive and negative features with this assignment. This is the first reported structure of TPK to which ATP or AMP is bound.

Mouse TPK exists as a homodimer in this space group (Fig. 3). No significant overall structural differences were found between this structure and the binary TPP and thiamine complexes of TPK with a RMSD of 0.38 Å and 0.39 Å of all main chain atoms, respectively. The products PPP and AMP together occupy a long groove, which is ~30 Å in length, 8 Å wide, and 8 Å deep. However, this binding groove is not closed, but is relatively open to the solvent. Two PPP molecules and two magnesium ions are bound in the structure. Similar to TPP in the TPP-complexed structure of TPK, PPP is found at the interface between the two subunits and adopts the F-conformation (Φ = 15° and Ψ = 74° in the A subunit and Φ = 27° and Ψ = 68° in the B subunit) (18, 27). The similarity of the PPP and TPP structures extends to the residues with which the respective products interact in the active site where all side chains are similarly placed in the PPP structure as found in the TPP structure (Fig. 4).

The one well-ordered AMP molecule bound in this structure adopts the C3’-endo anti-conformation for the adenosine group (Fig. 5). The major points of contact between the bound AMP molecule and TPK involve residues 25–26, 45–47, 99–101, 127–131, and 134–135. There are no direct hydrogen bonds between the enzyme and the adenine ring, but numerous van der Waals contacts are made. Two of these interactions are worth highlighting: one is between the main chain nitrogen atom of Arg51 and the N6 atom of the adenine and the other is between the main chain oxygen of Gly129 and the N1 atom. The location of these interactions and the lack of other specific interactions suggest that ATP may not be a specific substrate and that other nucleotide triphosphates can be utilized. The remaining interactions are largely hydrophobic contacts to the faces of the adenine ring and are provided by residues Gly127, Leu128, and Gly129. The main chain amide nitrogen atoms of Phe101 and Asn146 form hydrogen bonds to the O3’ and the O4’ atoms of the ribose ring, respectively. The negatively charged phosphoryl group interacts with the main chain amide of Gly47 and the side chain amide nitrogen of Asn25. Lastly, the bound Mg2+ ion coordinates with one of the phosphoryl oxygen atoms of AMP.

### Crystallographic statistics of data collection and refinement for mouse TPK-PPP-MgAMP complex structure

| Data sets                      | PPP-MgAMP-mouse TPK                        |
|--------------------------------|-------------------------------------------|
| Data collection                |                                           |
| Wavelength                     | 1.00 Å                                    |
| Mosaicity                      | 0.8                                       |
| Space group                    | P3_21                                     |
| Resolution (Å)                 | 30–2.5                                    |
| Cell constants                 |                                           |
| α, β, γ (°)                    | 89.36, 89.36, 141.43                      |
| α, β, γ (°)                    | 90, 90, 120                               |
| Measured reflections           | 87,425                                    |
| Unique reflections (%)         | 21,991                                    |
| Completeness (%)               | 94.5                                      |
| Rwork (%)                      | 11.7 (61.4)                               |
| Rfree (%)                      | 23.7 (25.2)                               |
| Refinement                     |                                           |
| Rwork / Rfree (%)              | 23.7 (25.2)                               |
| RMSD bond length (Å)           | 0.91                                      |
| RMSD bond angles (°)           | 1.64                                      |

*TABLE 1*
The binding of magnesium to the enzyme and products shifts the loop comprised of residues 71–75 (Fig. 6). The RMSD between the metal-bound conformation and other TPK structures for all atoms in this loop is 2.49 Å and 1.72 Å for just the main chain atoms. Both Mg²⁺ ions are octahedrally coordinated, but the nature of the interactions differs between the two subunits because of the difference in ligand occupancy in the B subunit. In the A subunit, the six coordinating interactions involve the α-phosphoryl of PPP, the phosphoryl group of AMP, and the side chain carboxylate groups of residues 46, 71, 73, and 100 (Fig. 7). The square plane of the octahedron is formed by the interactions contributed by the α-phosphoryl of PPP and the side chain carboxylates of Asp¹⁰⁰, Asp⁴⁶, and Asp⁷³. The phosphoryl group of AMP and the side chain carboxylate of Asp⁷¹ form the two axial positions of the coordination sphere.

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Evidence of PPP Formation from Kinetic Studies—The mechanism by which pyrithiamine inhibits the production of TPP by TPK was studied using both coupled enzymatic assays and by direct detection of the reaction products by mass spectrometry. AMP production was measured in the reaction mixture when thiamine or pyrithiamine was provided as the substrate for mouse TPK using a coupled enzyme assay system. The rate of AMP production with pyrithiamine was found to be 60 nmol/mg mouse TPK/min, which is about 80% of the rate obtained when thiamine was used as the substrate. Consequently, AMP was produced from ATP when thiamine or pyrithiamine was used as substrate for mouse TPK. However, it is possible that pyrithiamine binds to TPK, but promotes the hydrolysis of ATP to AMP and pyrophosphate. To rule out this possibility, we tested the pyrithiamine-containing reaction mixture for the existence of pyrophosphate using another coupled assay system. No pyrophosphate molecule was detected in the reaction mixture.

The enzymatic data support, but do not provide, positive evidence for the production of a pyrophosphorylated product of pyrithiamine, PPP. To detect the existence of PPP in the reaction mixture and further confirm what we observed in our structure, we performed mass spectrometry on the reaction products by mass spectrometry. AMP production was measured as the substrate for mouse TPK using a coupled enzyme assay system. No pyrophosphate molecule was detected in the reaction mixture.

Positive control experiments using thiamine in the reaction mixture were run in parallel. Thiamine was identified by a peak corresponding to an m/z ratio of 264.9 in positive ion detection mode in both the reaction mixtures with or without added enzyme. Operating in negative ion mode, an additional mass peak with an m/z ratio of 211.4 was present only in the reaction mixture with added enzyme. This peak corresponds to the mass of TPK carrying two negative charges. It is unclear whether these charge differences are related to inherent differences in the $p_K_a$ of the pyrophosphoryl groups of these compounds or whether slight differences in the final pH of the sample injected into the instrument influenced the observed charge distributions.

DISCUSSION

The structure of mouse TPK in a ternary complex with PPP and Mg$^{2+}$/AMP has been solved to 2.5-Å resolution by crystallization of a preformed equilibrium substrate/product mixture. This was a surprising result, because much of the available literature would suggest that pyrithiamine exerts its inhibitory effect without being an alternative substrate, such as that observed for oxythiamine (14). In addition to this structural result, we consistently detected the production of AMP in the enzymatic reaction mixture when pyrithiamine was provided as the substrate, and we detected the presence of PPP in the reaction mixture by performing mass spectrometry on the reaction products. Therefore, we conclude that similar to the action of oxythiamine, pyrithiamine is also a substrate for TPK and can be converted to its pyrophosphorylated product, PPP. In agreement with our results, Iwashima et al. (28) detected the presence of pyrithiamine pyrophosphate in rat brain tissue and in the reaction mixture catalyzed by partially purified pig TPK.

Although oxythiamine and pyrithiamine are structurally similar and can both be converted by TPK to their phosphorylated products (Ref. 14 and present work), the symptoms produced by them in animals are clearly different. Administration of oxythiamine to animals produces primarily metabolic aberrations such as lethargy and anorexia while the administration of pyrithiamine to animals produces neurological problems similar to Wernicke-Korsakoff Syndrome. The reasons for different physiological effects of the two analogs are currently unknown. It is known that OPP can inhibit the activity of TPP-dependent enzymes because the catalytically important 4$'$-amino group of TPP is replaced by a hydroxyl group in OPP that prevents the deprotonation of the C2 atom, thereby inactivating the coenzyme and, consequently, the enzyme (14, 15). Therefore, OPP would appear to disrupt cellular events primarily via inhibition of TPP-dependent enzymes and thereby produces mainly metabolic aberrations. However, thiamine pyrophosphate is also the precursor of TMP and TTP. TTP is believed to play important roles in brain and nerve tissues. Pyrithiamine pyrophosphate may disrupt thiamine phosphoester homeostasis through interference with the production or function of TMP/TTP, thereby producing mainly neurological disorders similar to Wernicke-Korsakoff Syndrome. The differential fate of OPP and PPP may be related to the chemical differences in thiazolium and pyridine rings. When we manually modeled PPP into the TTP site of the E1 component of the branched chain ketoacid dehydrogenase enzyme (PDB code 1OLS) we observed little difference in the way that PPP would be predicted to bind to TTP-requiring enzymes from a sterical point of view. However, there is no stabilizing force to keep PPP in the V-conformation. In pyrithiamine the carbon atom at the position equivalent to the C2 position of the thiazolium ring is not
Pyrithiamine as a Substrate for Thiamine Pyrophosphokinase

Independent of the nature of substrate addition and product release, the identification of Mg\(^{2+}\)/AMP and PPP in this structure allows us to ask whether a model for a ternary complex between the two natural substrates, thiamine and Mg\(^{2+}\)/ATP is feasible (Fig. 8). We find that such a complex is completely compatible with the current structural evidence. In this complex, the nucleophilic hydroxyl group of thiamine is in position (~3.8 Å in this modeled ground state structure) to attack the \(\beta\)-phosphoryl of ATP in a position opposing the bond to be broken when the AMP leaving group is formed. Stabilization of the transition state could be accomplished through interactions with the catalytic magnesium ion and Gln\(^{134}\), both of which are properly positioned to interact with the pentacoordinate intermediate that arises during phosphotransferase reactions. Additional evidence for the participation of residue 134 in the mechanism can be inferred from the fact that a hydrogen bond donor such as Gln or His is found in all known TPK enzymes at this position.

Mutagenesis experiments were recently reported that support the mechanism put forward here. These experiments probed the catalytic impact of residue exchanges at positions 71, 73, 74, 96, 99, 100, 131, and 133 (29). Mutation of the magnesium ligands Asp\(^{71}\), Asp\(^{73}\), and Asp\(^{100}\) to Asn all rendered the enzyme virtually inactive, consistent with their role in properly binding and modifying the ligand field of the metal ion for stabilization of the transition state. Mutation of the remaining residues was associated with modest changes in kinetic parameters that can be reconciled based on the ternary complexes presented here. The role of Gln\(^{96}\) has been discussed previously (17). Thr\(^{99}\) plays an important structural role to cap the \(\alpha\)-terminus of the \(\alpha\)-helix in which it resides, but its hydroxyl group is also 3.8 Å from the 3' ribose hydroxyl of AMP/ATP, and it is therefore not surprising that the mutation of this Thr to Ala would detrimentally affect the \(K_{m}\) for ATP. The roles of Arg\(^{131}\) and Asp\(^{133}\) are to stabilize the terminal phosphoryl group of either ATP or the pyrophosphorylated substrate. Arg\(^{131}\) interacts directly with the terminal phosphoryl of pyrithiamine pyrophosphate in this structure and with the terminal phosphoryl of thiamine pyrophosphate in prior structures. Arg\(^{131}\) is also predicted to interact with the \(\gamma\)-phosphoryl of ATP, based on our modeling experiment. Thus, it is not surprising that the \(K_{m}\) for ATP is adversely affected by the mutation of Arg\(^{131}\) to Gly. The role of Asp\(^{133}\) is to properly position Arg\(^{131}\) for these interactions, but it also forms an ion pair with the guanidine group of Arg\(^{131}\) and thus lowers the electrostatic nature of the interaction between Arg\(^{131}\) and the phosphoryl of ATP. This could explain why the mutation of Asp\(^{133}\) to Asn actually reduced the \(K_{m}\) for ATP, since Arg\(^{131}\) would carry a stronger formal positive charge in the context of the Asn\(^{133}\) mutation and thereby strengthen the binding interaction between ATP and the enzyme. The 2-fold reduction in \(k_{cat}\) associated with either mutation is likely because of subtle changes in the position of the ATP molecule or small changes in the stabilization of the transition state for the reaction.

\(^{3}\) J. Liu, unpublished data.

![FIGURE 8. Modeling of the catalytically competent ternary complex between Mg\(^{2+}\)/ATP and thiamine. Thiamine and ATP were modeled in this mouse TPK ternary structure by replacing PPP and AMP and manually altering the dihedral angles of the ligands. The protein structure was left unchanged. Amino acid residues that are involved in magnesium binding are colored light gray, while the magnesium ion is colored magenta.](image-url)
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One remaining enigma is the identification of a general base that would activate the thiamine hydroxyl group to an oxyanion. Our ternary complex does not provide an easily identified amino acid side chain that could function in this regard. One plausible suggestion is the ordered water molecule located between the side chain of Lys103 and the oxygen atom from which the proton would have been extracted (Figs. 4 and 7). Lys103 is a strictly conserved residue in all known TPK enzymes and is properly positioned for such an interaction. The proximity of the positively charged lysine residue to this water molecule suggests that the water could be bound in the hydroxide form prior to catalysis, thereby making the water molecule a good general base.

The structures of another pyrophosphokinase, HPPK, support an enzymatic mechanism in which the catalytic reaction occurs from within a ternary complex. One difference between HPPK and TPK is that two Mg$^{2+}$ ions are found in the HPPK active site, whereas only a single magnesium ion was observed in our current structure. The second Mg$^{2+}$ ion coordinates across the β- and γ-phosphoryl groups and includes the hydroxyl of the substrate within its coordination environment, thus directly lowering the pKₐ of the hydroxyl group and precisely positioning the oxyanion toward the β-phosphoryl group of ATP. Whether a second magnesium ion is required for Mg$^{2+}$/AMP binding and catalysis in TPK is not known, though there is sufficient space within the active site to accommodate a second Mg$^{2+}$ ion and perform the same catalytic function as observed for HPPK. Another difference between the two pyrophosphokinases is that even in the ternary complex, the TPK active site is fairly exposed to the solvent. In HPPK, the binding of the substrate to be pyrophosphorylated does not assemble the catalytic center well (31), but the binding of Mg$^{2+}$/ATP triggers dramatic conformational changes that seal the catalytic center (32). The high B factor for AMP in the A subunit of TPK and the mixed occupancy of HEPES and AMP in the B subunit suggests AMP is loosely bound to TPK. The open binding pocket and the lack of a number of highly specific interactions with the AMP is consistent with these findings and may indicate that adenose triphosphate is not a specific substrate for TPK.

Several studies over the years have investigated the nucleotide specificity of TPK and all studies have shown that the enzyme will utilize other nucleotide triphosphates as the pyrophosphate donor. In fact, a recent study suggested that UTP is a better substrate than ATP and the specific activity of TPK at fixed concentration of different nucleotides followed the following sequence UTP$>$ATP$>$CTP$>$GTP (29). This is an interesting finding and is consistent with our structure since the interactions surrounding the adenine ring in TPK would be better suited in terms of hydrogen bond donor and acceptor orientations for uracil or GTP. The fact that GTP is apparently a poor substrate is likely due to the steric restrictions that arise from the presence of the exocyclic amine group at the C2 position of guanine.

In conclusion, we have determined the three-dimensional structure of mouse thiamine pyrophosphokinase in ternary complex with AMP and pyritiamine pyrophosphate. This structure, combined with enzyme assays and mass spectrometry, provide clear evidence that pyritiamine can be utilized as an alternative substrate by thiamine pyrophosphokinase. These results suggest that the different phenotypes resulting from the administration of either pyritiamine or oxythiamine to animals result from different cellular fates for this pyrophosphorylated compounds, which are likely related to their differing physicochemical properties. The first ternary complex for TPK also provides an opportunity to understand the catalytic chemistry of the enzyme and the role of the catalytic magnesium ion. A surprising finding of this work was the lack of specificity with which the adenine ring of AMP interacted with the residues in the substrate binding site, which is consistent with prior work that showed the enzyme could utilize other nucleotide triphosphates with variable success to catalyze its reaction.

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