The Adenine Phosphoribosyltransferase from *Giardia lamblia* Has a Unique Reaction Mechanism and Unusual Substrate Binding Properties*

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Purine phosphoribosyltransferases catalyze the Mg$^{2+}$-dependent reaction that transforms a purine base into its corresponding nucleotide. They are present in a wide variety of organisms including plants, mammals, and parasitic protozoa. *Giardia lamblia*, the causative agent of giardiasis, lacks *de novo* purine biosynthesis and relies primarily on adenine and guanine phosphoribosyltransferases (APRTase and GPRTase) constituting two independent and essential purine salvage pathways. The APRTase from *G. lamblia* was cloned and expressed with a 6-His tag at its C terminus and purified to apparent homogeneity. Adenine and α-6-5-phosphoribosyl-1-pyrophosphate (PRPP) have $K_m$ values of 4.2 and 143 μM with a $k_{cat}$ of 2.8 s$^{-1}$ in the forward reaction, whereas AMP and PPi, have $K_m$ values of 87 and 450 μM with a $k_{cat}$ of 9.5 × 10$^{-2}$ s$^{-1}$ in the reverse reaction. Product inhibition studies indicated that the forward reaction follows a random Bi Bi mechanism. Results from the kinetics of equilibrium isotope exchange further verified a random Bi Bi mechanism in the forward reaction. In a mutant enzyme, F25W, with kinetic constants similar to those of the wild type and a tryptophan residue at the adenine binding site, the addition of adenine or AMP to the free mutant enzyme resulted in fluorescence quenching, whereas PRPP caused fluorescence enhancement. The dissociation constants thus estimated are 16.5 μM for adenine, 14.3 μM for AMP, and 83.0 μM for PRPP. PPi exerted no detectable effect on the tryptophan fluorescence at all, suggesting a lack of PPi binding to the free enzyme. An ordered substrate binding in the reverse reaction with AMP bound first followed by PPi, is thus postulated.

Purine phosphoribosyltransferases (PTases)$^1$ catalyze the Mg$^{2+}$-dependent replacement of the 1-pyrophosphate group from α-6-5-phosphoribosyl-1-pyrophosphate (PRPP) by a purine base to form the corresponding purine nucleotide 5’-monophosphate. They play major roles in purine salvage among most living organisms. In mammals and many other organisms, purine nucleotides can be generated via a *de novo* synthetic pathway as well as several purine salvage pathways incorporating preformed purine bases and nucleosides into the purine nucleotide pool. For parasitic protozoa, however, there is no *de novo* synthesis of purine nucleotides (1). Most of them rely on multiple purine salvage pathways to replenish their purine nucleotides (2). A simultaneous inhibition of all the major purine salvage enzymes will be necessary for depleting purine nucleotides from these organisms. However, among certain parasitic protozoa, such as *Giardia lamblia*, an anaerobic binucleate flagellated protozoan known to cause giardiasis in mammals (3), and *Trichomonas fetus*, which causes embryonic death and infertility in cows (4), their purine salvage pathways are quite simple. *T. fetus* depends primarily on a single salvage enzyme, hypoxanthine-guanine- xanthine phosphoribosyltransferase (HGXPRTase), to supply its purine nucleotides (5). *T. fetus* HGXPRTase has been analyzed thoroughly in our laboratory and proven to be a *bona fide* target for anti-trichomonas chemotherapy and a successful example of rational drug design (6–8). *G. lamblia* relies on two salvage enzymes, guanine phosphoribosyltransferase (GPRTase) and adenine phosphoribosyltransferase (APRTase) to replenish its purine nucleotide pool (9). There is little interconversion between guanine and adenine nucleotides in *G. lamblia* (1, 9); both the GPRTase and the APRTase in this organism are thus potential targets for anti-giardiasis chemotherapy. *G. lamblia* GPRTase has been characterized extensively in our laboratory in recent years (10–13) and has been demonstrated, by down-regulating GPRTase gene expression, to perform an essential function in the organism; it is thus a target for anti-giardiasis drug design (13). Our interest is now focused on dissecting the structure and function of *G. lamblia* APRTase as another potentially interesting target for anti-giardiasis chemotherapy. Because deficiency in human APRTase is known to cause a rare form of kidney stones (2,8-dihydroxyadenine urolithiasis derived from oxidation of excessively accumulated adenine by xanthine oxidase) (14, 15), specific inhibitor design against only the parasite enzyme based on an in-depth understanding of the structure-function of the latter would be an important approach for a potentially useful therapy.

In our current study we have cloned, expressed, and purified the recombinant *G. lamblia* APRTase in substantial quantities. Crystallographic analysis of its three-dimensional structure (16) indicates a symmetric homodimer of monomers, each built around a Rossman fold core with the active site capped with a small hood, as has been also observed in the three-dimensional structures of APRTases from *Leishmania donovani* (17) and
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**Saccharomyces cerevisiae** (18). Unique structural features of *G. lamblia* APRTase include a *cis* peptide bond, Glu¹⁸³-Ser²⁹² at the PP, binding site in the enzyme-9-deazaadenine-MgPRPP complex that is in a *trans* configuration in the enzyme-9-deazaadenine-SO₄ complex. There is thus most likely a PRPP-induced structural change that converts the Glu¹⁸³-Ser²⁹² peptide from *trans* to *cis* configuration for an activated PP, binding site. The nearest overall three-dimensional structural homologue of *G. lamblia* APRTase turns out to be the orotate phosphoribosyltransferase (OPRTase) from *Salmonella typhimurium* (19, 20), suggesting a common path of evolution shared by these two enzymes separate from the other purine PRTases. The PP, binding site in OPRTase-OMP complex, Arg⁶⁹⁹-Lys¹⁰⁰ did not, however, stand out in a *cis* configuration in the previous study (20).

The HGPRTase-catalyzed reactions have been investigated extensively and shown to follow an ordered Bi Bi mechanism (21–25). Without an exception, PRPP is always bound to the free enzyme first, followed by the binding of the purine base, whereas PP, is released prior to the release of purine nucleotide upon completion of the reaction (21–24). The OPRTase-catalyzed reaction follows, however, a random Bi Bi mechanism (26). Little study has been performed on the mechanisms of APRTase-catalyzed reactions until the recent study of the enzyme from *L. donovani* (27). The *L. donovani* APRTase exhibits a strictly ordered mechanism with PRPP being added first and AMP being the last product released. However, the *L. donovani* APRTase is atypical in structure, having an additional 50 amino acids at the N and C termini relative to the *G. lamblia* enzyme as well as all the other APRTases with known primary structures (17). In our current study, we have determined the basic kinetic constants of *G. lamblia* APRTase-catalyzed reactions and present evidence of a random Bi Bi pattern of substrate binding in the forward reaction. A sequential substrate binding in the reverse direction with AMP bound to the free enzyme prior to PP, binding was postulated for this enzyme from fluorescence quenching studies. Apparently, the mechanism of Giardia APRTase-catalyzed reactions differs from those of Leishmania APRTase and Salmonella OPRTase.

**MATERIALS AND METHODS**

**Chemicals and Reagents**—All of the chemicals used in the present study, including adenine, AMP, PP₃, xanthine oxidase, and the tetrasodium salt of PRPP, were purchased from Sigma and are of the highest purity available. [8-¹⁴C]Adenine was obtained from Moravek Biochemicals, Inc.

**Cloning of the Full-length APRTase Gene from G. lamblia**—Primers corresponding to the 5' and 3' ends of the APRTase gene (5'-GCTTATAGGACCAGTTGGCCG-3' and 5'-CTCGACACCTACTAGCCCTTTATCTTCTCTGAG-3') were designed using BLAST, Clustal W, version 1.4, from the sequence information derived from the *G. lamblia* genome project at the Marine Biological Laboratory (Woods Hole, MA). Polymerase chain reactions (PCR) were performed using *G. lamblia* genomic DNA as template (28). The full-length APRTase genomic DNA was cloned, sequenced, and deposited in the GenBank™ data base (accession number AF378363). The full-length APRTase genomic DNA was cloned into the pGEM T-easy vector (Promega), excised, and inserted into a pBac expression vector (29) with an added sequence encoding a C-terminal His tag. The recombinant plasmid (pBaprt) was transformed into *Escherichia coli* BL21 cells (Stratagene), and the recombinant APRTase protein was expressed in a low-phosphate induction medium (29).

**Purification of the His-tagged Recombinant APRTase Protein**—E. coli BL21 cells transformed with pBaprt grown in low-phosphate induction medium at 37 °C for 24 h (29) were sonicated in the lysate buffer (50 mM HEPES, pH 6.0, 300 mM NaCl, 8 mM MgCl₂, 20 mM imidazole) with the protease inhibitor mixture (Roche Molecular Biochemicals). The overexpressed recombinant APRTase protein with a C-terminal His tag was purified by the lysate using Ni-NTA-agarose (Qiagen) by the following procedure. A sample of 15 ml of clear cell lysate was combined with 5 ml of Ni-NTA-agarose suspension, mixed gently at 4 °C overnight, and then washed with 50 ml of the 50 mM HEPES buffer, pH 6.0, with 20 mM imidazole and 300 mM NaCl. The His-tagged protein was eluted with 200 mM imidazole in the same HEPES buffer. The purity of the protein was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**Enzyme Assays**—All APRTase assays were performed in 100 mM Tris-HCl, pH 7.4, and 12 mM MgCl₂ at 37 °C. Kinetic data were collected using a Beckman DU-640 spectrophotometer equipped with a kinetics accessory. The formation of AMP was monitored spectrophotometrically at 260 nm, which is the wavelength at which the difference in absorbance between AMP and adenine has an extinction coefficient of 1600 M⁻¹ cm⁻¹. The final volume of assay mixture, containing various amounts of the substrates, was 0.5 ml.

For the reverse reaction, adenine production was monitored indirectly using a xanthine oxidase-coupled spectrophotometric assay adapted recently in our laboratory. An excessive amount (1 unit) of xanthine oxidase was added to facilitate the formation of 2,8-dihydroxyadenine from adenine (30). The production of 2,8-dihydroxyadenine was monitored spectrophotometrically at 301 nm with a differential extinction coefficient of 15,200 M⁻¹ cm⁻¹. Because of the nearly 10-fold higher extinction coefficient compared with that used in monitoring the forward reaction, this newly devised assay has, by our knowledge, the highest sensitivity in detecting product formation in the APRTase-catalyzed reverse reaction.

**Kinetic Data Analysis**—Initial velocity data were collected at 7–8 different concentrations of the first substrate at different fixed concentrations of the second substrate. Kinetic constants were determined from Lineweaver-Burk plots of the initial velocity data, using weighted linear regression. For product inhibition studies, initial velocity results were collected from 7–8 different concentrations of the first substrate at a saturating concentration of the second substrate and different fixed concentrations of a product. Initial rate data were fitted to Equations 1–5 using kinetics software from BioMetallics, Inc. (kcat) and SPSS, Inc. (Sigma Plot 2000 with kinetics module). Kinetic constants were calculated using a weighted linear regression.

For the sequential mechanism,

\[ v = V_{\text{max}}AB/K_A + K_B + KA + AB \]  
(1 Eq.)

For equilibrium ordered,

\[ v = V_{\text{max}}AB/K_A + KA + AB \]  
(2 Eq.)

For competitive inhibition,

\[ v = V_{\text{max}}S[K_A(1 + I/K_A) + S] \]  
(3 Eq.)

For noncompetitive inhibition,

\[ v = V_{\text{max}}S[K_A(1 + I/K_A) + S(1 + I/K_A)] \]  
(4 Eq.)

Kinetic data were fitted globally in a random fashion to the correct equilibrium model to obtain the kinetic parameters. The best fits were determined by the relative fit error and errors in the constants. The nomenclature is that of Cleland (31): \( v \), initial velocity; \( V_{\text{max}} \), maximum velocity; \( A \), concentration of substrate A; \( B \), concentration of substrate B; \( K_A \), \( K_B \), for A; \( K_{AI} \), \( K_{AI} \), for B; \( K_{AI} \), \( K_{AI} \), for product Q; \( S \), substrate concentration; \( K_a \), Michaelis constant; \( K_{AI} \), \( K_{AI} \), slope and intercept inhibition constant; \( I \), inhibitor concentration. \( K_{AI} \) values were determined using the method outlined by Segel (32) for a rapid equilibrium random Bi Bi system. The \( K_{AI} \) value of the reaction was estimated from the kinetic constants by Haldane’s relationship (32). The kinetic rate constants were calculated from the kinetic parameters where possible using the following equations and nomenclature of Cleland (31).
Fig. 1. Alignment of APRT sequences. Conserved residues are shaded in gray. The blue bar indicates the position of the flexible loop, the red bar indicates the PRPP binding motif, and the green star indicates the catalytic residue (based on the structure of the L. donovani APRTase (17)). Sequences were aligned using ClustalW 1.4. GenBankTM data base entries shown are (accession numbers in parentheses): G. lamblia (378363), S. cerevisiae (1703347), L. donovani (409142), Homo sapiens (410074), S. aureus (22590431), P. aeruginosa (416330), M. pneumoniae (6686265), Mus musculus (114075), H. pylori (3182011), H. influenzae (1168477), E. coli (1104073), D. melanogaster (479995), B. subtilis (6647401), and A. thaliana (249931).

exchanges were carried out between the two product-substrate pairs, AMP-adenine and PP1-PRPP. The initial ratio of concentrations between the product and substrate in each pair was fixed by estimating their equilibrium concentrations, taking the ratio of their isotope exchange to assure that all substrates and products were at their equilibrium concentrations. Reactions were equilibrated at 37 °C for 2 h prior to initiating the purified recombinant, G. lamblia APRTase, was added to a 100-μl reaction mixture containing 0.1 M Tris-HCl, pH 7.4, and 12 mM MgCl2. The substrate/product pair reaction mixture was quenched by adding 5 mM EDTA (pH 8.0), and the final reaction mixture was spotted on a polyethyleneimino-cellulose plate and developed for 8 cm in 5 mM ammonium acetate at pH 4.5 (22). The equilibrium isotope exchange reaction was initiated at the end of 2-h incubation by adding [8-14C]adenine (0.005 μCi) to the equilibrated reaction mixture. After 4 min at 37 °C, each of the exchange reactions was quenched by adding 5 μl of 0.5 M ammonium acetate at pH 8.0, and 20 μl of the reaction mixture was spotted on a polyethyleneimino-cellulose plate and developed for 8 cm in 5 mM ammonium acetate at pH 4.5 (22). The Rf values under these conditions were 0.0 for AMP and 0.32 for adenine. Radiolabeled spots were imaged using a PhosphorImager (version 5.2; Amersham Biosciences). Rates for the equilibrium isotope exchanges were calculated and expressed as percent conversion and plotted against the concentration of the variable product-substrate pair as described by Purich and Allison (33).

**Unique Reaction Mechanism of G. lamblia APRTase**

The full-length cDNA encoding G. lamblia APRTase was cloned from a G. lamblia genomic DNA library through the use of specifically designed PCR primers by sequence alignments of other known APRTases and with information from the G. lamblia genome project (Marine Biological Laboratory). The full-length genomic DNA thus cloned was sequenced (GenBankTM accession number AF375363), and the protein sequence thus derived from it, which contains no tryp-
tophan residue, was aligned with those of other APRTases from other living organisms (Fig. 1). Results from Fig. 1 indicate the percentages of protein sequence identity (similarity) with the APRTases from S. cerevisiae 32 (51), L. donovani 21 (39), human 37 (56), S. aureus 34 (52), Pseudomonas aeruginosa 29 (48), Mycoplasma pneumoniae 27 (49), Mus musculus 36 (56), Helicobacter pylori 33 (53), Haemophilus influenzae 33 (55), E. coli 34 (53), Drosophila melanogaster 32 (52), Bacillus subtilis 33 (51), and Arabidopsis thaliana 31 (49). Through the alignments with APRTases from L. donovani and yeast, which have their crystal structures resolved by x-ray diffractions (17, 18), we were able to tentatively identify in G. lamblia APRTase has a predicted molecular mass of 20,370 kDa, which was verified by mass spectrometry (data not shown).

**Steady-state Kinetics of G. lamblia APRTase-catalyzed Reactions**—The steady-state kinetics of AMP synthesis and pyrophosphorylation catalyzed by G. lamblia APRTase were analyzed. Double-reciprocal plots of initial reaction velocities versus the concentrations of one substrate at a series of fixed concentrations of the other substrate were analyzed for both forward and reverse reactions catalyzed by G. lamblia APRTase (Fig. 2). The C-terminal His-tagged recombinant APRTase protein was expressed in E. coli BL21 cells using a low-phosphate induction system. The purified His-tagged recombinant APRTase protein (Fig. 2) has an estimated molecular mass of 20,370 kDa, which agrees well with the predicted molecular weight of 20,370 from the encoding cDNA.

**Product Inhibition in the G. lamblia APRTase-catalyzed Reactions**—To determine whether the sequential mechanism of G. lamblia APRTase-catalyzed reaction is ordered or random, product inhibition was analyzed for the forward phosphoribosyltransferase reaction. PPi was found to be noncompetitive with respect to PRPP with an extremely high \( K_{\text{m,PPi}} \) value of 1787 ± 157 \( \mu \text{M} \) (Fig. 4A). PPi demonstrated insufficient inhibition within the limitation of its solubility with respect to adenine.

**Equilibrium Isotope Exchange**—Fig. 5 shows the effect of increasing PPi-PRPP concentrations on the rate of AMP-adenine isotope exchange. The rate of exchange reaches a maximum at a 1:1 ratio of the PPi,PRPP and AMP-adenine pairs.
There is no depression in the exchange rate upon further increases in the PPi-PRPP concentration. Because the exchange between AMP and adenine requires that there be a form of the enzyme that could absorb the labeled species, the unchanged maximum rate of exchange, despite the continued increase in the PPi-PRPP concentration, suggests the presence of a constant level of such an enzyme form under these conditions. In the case of adenine, this constant level of available enzyme form must be the combined population of free enzyme and the PPi-PRPP complex, which means that adenine can bind to both forms. Thus the forward reaction proceeds with adenine and PPi binding to the active site in a random order. The order of substrate binding in the reverse direction could not be determined because of the less than saturating level of PPi and PRPP that could be reached in the study.

**Substrate Binding to G. lamblia APRTase F25W Mutant**—To further investigate the reaction mechanism catalyzed by *G. lamblia* APRTase, a tryptophan residue was engineered into the purine binding pocket of the *G. lamblia* APRTase active site to replace the phenylalanine residue (F25W), which was demonstrated in the crystallographic analysis to form a stacking interaction with the purine ring (16). The mutation was thus designed for monitoring the tryptophan fluorescence quenching as an indication of adenine or AMP binding to the active site. This F25W mutation does not significantly affect the enzyme activity and the kinetics of the catalyzed reactions. The *Kd* value for adenine in the mutant enzyme-catalyzed reaction was estimated to be 8 µM, which is close to the wild-type value of 4.2 µM (see Table I). The addition of AMP to the mutant enzyme quenched its fluorescence, and the quenching effect was dependent on the increasing concentrations of AMP (Fig. 6A) giving an estimated *Kd* = 14.3 ± 1.9 µM. The results confirm the capability of AMP in binding to the free enzyme and the further identification of residue 25 in the binding site for AMP. Addition of adenine to the free mutant enzyme also quenched the fluorescence (Fig. 6B). The dissociation constant thus estimated has a value of 16.5 that could absorb ± 0.79 µM, which is extremely close to that of AMP, suggesting that adenine and AMP are both capable of binding to the active site in a free APRTase by a similar mechanism. Upon the addition of PRPP to the free mutant enzyme, however, the fluorescence signal increased, indicating a possible conformational shift in the active site region freeing the Trp25 residue from its original confinement (Fig. 6C). The PRPP binding site identified in the crystal structure of *G. lamblia* APRTase-9-deazaadenine-Mg-PRPP complex indicates that it is separated from that of 9-deazaadenine with only the guanidino group of Arg63 shared by both substrates through hydrogen bonding (16). An estimation of the dissociation constant for PRPP based on its concentration-dependent fluorescence enhancement (Fig. 6C) results in a *Kd* = 83 ± 3.4 µM, which is reasonably close to its *Km* value of 143 µM in the wild-type enzyme catalyzed reaction. PPi did not affect the fluorescence signal from the free mutant enzyme up to its limitation of solubility at 1.2 mM. Although it is possible that PPi binding to the free enzyme may not elicit any fluorescence change in the mutant enzyme, the lack of any PPi effect on the fluorescence enhancement induced by PRPP or fluorescence quenching caused by adenine (data not shown) suggests that its failure in binding to any of the three forms of APRTase in an appreciable way may provide the most likely explanation; this is also in good agreement with the data from the previous product inhibition study. This conclusion, coupled with indication of AMP binding to the free enzyme from product inhibition and fluorescence quenching, suggest that a reasonable explanation would be that AMP binds to the free enzyme first, followed by PPi binding in the reverse reaction. The mechanism of the reverse reaction could be thus in a pattern of ordered-on and random-off. This conclusion, depicted in the scheme presented in Fig. 7, calls for an ordered-random Bi Bi mechanism for *G. lamblia* APRTase-catalyzed forward reaction. The rate constants for individual steps in the reaction, calculated from the Michaelis constants listed in Table I, indicate that the release of AMP from the enzyme complex is the rate-limiting step (Fig. 7).

**DISCUSSION**

In the present investigation, we identified and isolated the encoding gene of *G. lamblia* APRTase and expressed and purified a significant amount of the recombinant enzyme protein to apparent homogeneity. Steady-state kinetic analysis of the enzyme-catalyzed reactions provided kinetic constants. From the *Km* and *kcat* values presented in Table I, the >3 orders of magnitude higher values for the forward reaction suggest strongly that the primary function of APRTase in *G. lamblia* cells is to synthesize AMP from adenine and PRPP, most likely to fulfill the need of adenine nucleotides.

Product inhibition and equilibrium isotope exchange studies indicate that the APRTase-catalyzed forward reaction follows a random Bi Bi mechanism of substrate binding. This is in contrast to all other known purine phosphoribosyltransferases, for which the reaction mechanisms have been determined to be ordered Bi Bi with PRPP binding to the free enzyme first, followed by the purine base, whereas the products are released in the order of PPi first and the purine nucleotide second (10, 21, 23, 24). Binding of PPi and adenine to the free APRTase was also demonstrated by their fluorescence enhancing and quenching effects on the F25W mutant enzyme. Binding of adenine to the active site in free enzyme was further demonstrated in the crystallographic analysis, in which the crystal form of *Giardia* APRTase-9-deazaadenine-SO4 complex was obtained and its structure resolved (16).

A recent kinetic study on *L. donovani* APRTase-catalyzed reactions reported a strictly ordered Bi Bi reaction mechanism like those reported previously for the HGPRTases catalyzed reactions (27). It was a somewhat surprising finding, because crystals of *Leishmania* APRTase-adenine complex were obtained previously and used for structural determination, suggesting that adenine is capable of binding to the free enzyme (17). Product inhibition indicated a noncompetitive pattern of PPi inhibition *versus* both adenine and PRPP (27), suggesting...
possible formation of the dead-end complex E-adenine-PPi, which again indicates probable adenine binding to the enzyme without the a priori presence of PRPP. Overall, a possible random substrate binding model in the L. donovani APRTase-catalyzed forward reaction has not been, in our opinion, totally ruled out at the present time.

The only other phosphoribosyltransferase catalyzed reaction with a demonstrated random Bi Bi mechanism of substrate binding is the OPRTase from S. typhimurium. The mechanism of reaction, determined through product inhibition and kinetic isotope studies (26, 34), showed that OMP was a competitive inhibitor of both orotate and PRPP, in perfect agreement with our findings on the Giardia APRTase-catalyzed forward reaction. Interestingly, data from structural analysis of G. lamblia APRTase suggested that its nearest structural relative is not any of the other purine PRTases with known crystal structures but the OPRTase from S. typhimurium (16). Apparently, these two enzymes have been in the same path of evolution, distinctive from that of the other purine PRTases.

There is, however, also some discrepancy in the product inhibition data between Salmonella OPRTase and Giardia APRTase. In the former case, PPi was found to be competitive versus PRPP and noncompetitive versus orotate, suggesting the formation of an E-orotate-PPi dead-end complex (26). Our results on the Giardia APRTase indicated a weak noncompetitive pattern of inhibition for PPi versus PRPP (Fig. 4A). Insufficient inhibitory effect was registered for PPi versus adenine, indicating an apparent absence of the anticipated dead-end E-adenine-PPi complex. This is attributed to the inability of PPi to bind to the E-adenine complex (see below). Subsequent fluorescence study of the F25W mutant enzyme showed that AMP causes fluorescence quenching in the free mutant enzyme with an estimated $K_d$ value, similar to its $K_m$ value, whereas PPi exerted no detectable effect on the protein fluorescence of the free mutant enzyme up to its saturating concentration of 1.2 mM. PPi is apparently incapable of binding to the free enzyme also. Thus the most likely mechanism of substrate binding in the reverse reaction is that AMP binds to the free enzyme first followed by PPi binding. This postulated "ordered on" in the reverse direction leads to the conclusion that the forward reaction proceeds with a mechanism of random Bi Bi with random substrate binding but ordered product release (see Fig. 7).

This unique mechanism of reaction may find its structural basis in the two crystal structures of APRTase, the APRTase-9-deazaadenine-SO4 and APRTase-9-deazaadenine-PRPP complexes (16). One major structural distinction between these two complexes is in the conformation of dipeptide Glu61–Ser62 at the PPi binding site. The non-proline peptide bond is in a cis configuration in the APRTase-9-deazaadenine-PRPP complex and constitutes the functioning PPi binding site. The change from the trans configuration in the APRTase-9-deazaadenine-SO4 complex without PRPP (16). In the trans configuration, the side chain of Glu61 extends into the PRPP/PPi binding pocket presenting a barrier to PPi binding. The change from trans to cis configuration is apparently induced by PRPP binding to the
enzyme. It moves the Glu61 residue out of the way to allow the interaction between PPi and Ser62–Arg63 for effective binding (16). Presumably, interaction between the ribotide-bound Mg\textsuperscript{2⁺} and the Glu61 carboxylate oxygen through a water molecule leads to the configuration change (16). In the enzyme-adenine complex, and probably also in the free enzyme, where this peptide bond assumes the trans configuration, binding to PPi would be unlikely (16). It explains the failure of PPi in binding to the free enzyme or forming the E-adenine-PPi dead-end complex. It is not yet known whether binding of AMP to APRTase will bring about the same change of peptide configuration, because the crystal structure of APRTase-AMP complex is not yet available. But, by assuming a similar binding pattern of the 5′-ribotide-Mg\textsuperscript{2⁺} moiety in PRPP and AMP within the same enzyme active site, it is likely that a similar trans to cis isomerization of the Glu61–Ser62 dipeptide will be induced. This explains also the requirement of a priori AMP binding before PPi can bind.

The trans isomer of a non-proline peptide bond has been found to be favored over the cis isomer by 2.6 kcal/mol (35). The occurrence of non-proline cis peptide bonds in proteins is quite rare. A recent tabulation done in 1990 (35) among proteins with known structures indicated that of 31,005 peptide bonds, only 17 (0.05%) were of the cis configuration. None of the 17 cis peptides, as far as we are aware, has been assigned to any specific functional significance in the protein. Our previous structural analysis of a related enzyme, the HGXPRTase from T. fetus, done in 1996 (36), demonstrated in the crystal struc-
ture of the enzyme-GMP complex a non-proline cis dipeptide Leu$^{56}$–Thr$^{57}$ at the location corresponding to the PP$_i$ binding site. Since then many other purine PRPTases have been identified as possessing a cis peptide at the PP$_i$ binding site (12) (37–41). A trans to cis to trans cycle of isomerization was proposed by Heroux et al. (41), in which the cis form may be required for binding of PRPP or PP$_i$ whereas the trans form may be needed for expulsion of PRPP or PP$_i$. The OPRTase from S. typhimurium was found to proceed by a random Bi Bi mechanism, both configurations of this dipeptide may not have much bearing to the presence of both forms in the same complex suggests that the enzyme-PRPP-orotate complex (20). A more recent study indicates that this dipeptide is found in a cis configuration in another crystallized enzyme-orotate-PRPP complex. The presence of both forms in the same complex suggests that the configuration of this dipeptide may not have much bearing to PRPP or PP$_i$ binding. Because this enzyme-catalyzed reaction was found to proceed by a random Bi Bi mechanism, both configurations of the dipeptide could be capable of binding to PP$_i$. Further investigation will be needed to clarify this interesting point.

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