

Biochemistry. Author manuscript; available in PMC 2008 October 14.

Published in final edited form as:

Biochemistry. 2006 December 19; 45(50): 14933-14943. doi:10.1021/bi061802v.

# Substrate recognition by the hetero-octameric ATP phosphoribosyltransferase from *Lactococcus lactis*†

Karen S. Champagne<sup>‡,§</sup>, Elise Piscitelli<sup>||</sup>, and Christopher S. Francklyn<sup>‡,||,\*</sup>

‡ Departments of Microbiology & Molecular Genetics, University of Vermont, B403 Given Building, 89 Beaumont Ave, Burlington, VT 05405

|| Department of Biochemistry, Health Sciences Complex, University of Vermont, B403 Given Building, 89 Beaumont Ave, Burlington, VT 05405

### Abstract

Two families of ATP phosphoribosyl transferases (ATP-PRT) join ATP and 5-phosphoribosyl-1 pyrophosphate (PRPP) in the first reaction of histidine biosynthesis. These consist of a homohexameric form found in all three kingdoms, and a hetero-octameric form largely restricted to bacteria. Hetero-octameric ATP-PRTs consist of four HisG<sub>S</sub> catalytic subunits related to periplasmic binding proteins, and four HisZ regulatory subunits that resemble histidyl-tRNA synthetases. To clarify the relationship between the two families of ATP-PRTs, and among phosphoribosyltransferases in general, we determined the steady state kinetics for the heterooctameric form, and characterized the active site by mutagenesis. The  $K_m$  PRPP (18.4  $\pm$  3.5  $\mu$ M) and  $k_{cat}$  (2.7 ± 0.3 sec<sup>-1</sup>) values for the PRPP substrate are similar to those of hexameric ATP-PRTs, but the  $K_m$  for ATP (2.7  $\pm$  0.3 mM) is 4-fold higher, suggestive of tighter regulation by energy charge. Histidine and AMP were determined to be non-competitive ( $K_i = 81.1 \mu M$ ) and competitive ( $K_i$ = 1.44 mM) inhibitors, respectively, with values that approximate their intracellular concentrations. Mutagenesis experiments investigating the side chains recognizing PRPP showed that 5' phosphate contacts (T159A and T162A) had the largest (25- and 155-fold) decreases in k<sub>cat</sub>/K<sub>m</sub>, while smaller decreases were seen with mutants making cross subunit contacts (K50A and K8A) to the pyrophosphate moiety, or contacts to the 2' OH. Despite their markedly different quaternary structures, hexameric and hetero-octameric ATRP-PRTs exhibit similar functional parameters, and employ mechanistic strategies reminiscent of the broader PRT superfamily.

ATP phosphoribosyltransferase (ATP-PRT; E. C. 2.4.2.17) catalyzes the first and highly regulated step of histidine biosynthesis, which involves nucleophillic attack of the N1 of ATP on C1′ of 5-phosphoribosyl- 1 pyrophosphate (PRPP) to form N-1-(5′-phosphoribosyl)-ATP (PR-ATP, Figure 1A) (1). The resulting product is converted through an additional nine reactions into histidine by a pathway that is regulated both by its end product and by AMP and ADP (2). The tight regulation of this pathway (reviewed in (3)), reflects the high energetic costs associated with histidine synthesis, and the need to dramatically slow pathway flux when histidine is present in the growth media (4). Superimposed on top of metabolic control of

<sup>&</sup>lt;sup>†</sup>This work was supported by N.I.H. grant GM54899 (CSF) and the Department of Energy Experimental Program to Stimulate Competitive Research (DOE-EPSCOR)

<sup>\*</sup>To whom correspondence should be addressed: Christopher Francklyn, Ph.D., Department Biochemistry, University of Vermont College of Medicine, 89 Beaumont Ave, phone: 802-656-8450, fax: 802-862-8229, email: Christopher.Francklyn@uvm.edu. \*Current address: Department of Molecular, Cell, and Developmental Biology, 406 Sinsheimer Labs, University of California, Santa Cruz, Santa Cruz, CA 95064

histidine biosynthesis is genetic regulation of the expression of histidine biosynthetic enzymes, by use of the classic attenuation mechanism (reviewed in (5)).

ATP-PRT is a member of the phophoribosyltransferase (PRT) superfamily of enzymes, all of whom share a common chemistry that involves transfer of the phosphoribosyl group to a nucleotide base or, in the case of glutamine phosphoribosyl pyrophosphate amidotransferase, to free ammonia generated on the enzyme (6). PRTs are found in many essential pathways, including biosynthesis and salvage of nucleotides, and the synthesis of cofactors and amino acids (7). There are at least four different structurally unrelated subfamilies of PRTs. Type I PRTs, which include the adenine, orotate (OPRT), and hypoxanthine/guanine PRTs (HGPRT), are composed of four different domains, including a core region that binds both the PRPP and the nucleotide substrate, a hood domain that recognizes the nucleotide substrate, a flexible loop that closes over PRPP, and a C-terminal arm that provides dimerization for some PRTs (8). Considerable research attention has focused on the type I PRTs, as the inherited Lesch/Nyan syndrome and orotic aciduria involve respective defects in the HPRT and orotate PRTs (7). Other type I PRTs represent targets for therapeutics directed against malaria, Chagas disease, and other pathogenic eukaryotes. Notably, type I PRTs share the PRPP binding motif, a thirteen residue signature sequence that is important for substrate recognition (9). The type II and type III enzymes are represented by quinolinate (QPRT) and anthranilate PRT, respectively, which participate in NAD+ and tryptophan biosynthesis (10,11).

ATP-PRTs represent a fourth class of PRTs, and comprise two different subfamilies with distinctly different quaternary structures. The hexameric ATP PRTs, referred to as HisG<sub>I</sub> or long form, are found in enteric bacteria and lower eukaryotes (12). Each HisG<sub>I</sub> subunit is composed of three domains (13,14). The first two constitute a mixed  $\alpha/\beta$  bilobal catalytic domain that resembles proteins of the periplasmic binding protein family (15), while the third domain provides a binding site to allow feedback inhibition by histidine (13). The two different HisG<sub>L</sub> structures available include a histidine/AMP inhibited form from *Mycobacterium* tuberculosis (13), and a enzyme product complex between PR-ATP and the enzyme from Escherichia coli (14). Enzymes from the second ATP-PRT family are hetero-octamers, composed of four HisGs subunits and four HisZ regulatory subunits (Figure 1B) (16,17). The latter are structurally related to the catalytic domain of histidyl-tRNA synthetase, and provide a regulatory domain to compensate for the absence of the C-terminal domain found in HisG<sub>I</sub>. Notably, both subunits are required to form a functional complex (17). Two structures of the hetero-octameric ATP-PRT have been reported, a histidine-inhibited complex of the enzyme from Thermatoga maritima (18), and ATP-activated and PRPP-bound forms of the enzyme from Lactococcus lactis (19).

The sequence conservation between  $\operatorname{HisG}_S$  and  $\operatorname{HisG}_L$  indicates a conserved active site, and suggests a similarly conserved reaction mechanism (Figure 2). Early work characterizing the steady state kinetics of the long form indicated that the reaction proceeds by an ordered Bi-Bi mechanism with ATP leading (20). Another study involving determination of kinetic isotope effects of the  $\operatorname{HisG}_L$  enzyme provided evidence that the ATP-PRT transition state has ribooxocarbenium character (21). In contrast, the kinetic properties of the hetero-octameric form have not yet been characterized. As part of efforts to compare the structure and function of the hexameric and hetero-octameric versions of ATP-PRT, we report here the steady state kinetics of the hetero-octameric  $\operatorname{HisG/HisZ}$  enzyme from  $\operatorname{Lactococcus lactis}$ , and a mutational analysis of the contacts between PRPP and the ATP-PRT based on the structure of the activated form of the complex (19). Analysis of the resulting mutant proteins highlights the importance of active site contacts with PRPP on ground state binding, as well as the interdependence of the recognition of PRPP, ATP and the inhibitor histidine.

### EXPERIMENTAL PROCEDURES

### Construction and purification of mutant proteins

Mutant versions of the *L. lactis* HisZ/HisG ATP-PRT were constructed in the pQE30 expression construct background (17). The T159A, T162A, S140A, K8A, K50A and D155A mutations were introduced by use of the QuikChange® mutagenesis procedure (Stratagene). In all cases, the double stranded primers were 45 nucleotides in length. After mutagenesis, the presence of the directed mutations and the absence of unprogrammed mutations were confirmed by sequencing of the entire genes. The wild type and all mutant proteins were expressed and purified from *E. coli* over-expression strains by use of a previously published protocol featuring the three-column sequence of Ni-NTA, Superdex 200, and hydroxyapatite chromatography (16). Pooled fractions from the final hydroxyapatite column were concentrated to ~6 mg/mL, dialyzed into storage buffer (50 mM Na<sub>2</sub>PO<sub>4</sub> pH 7.5, 300 mM KCl, 10% (v/v) glycerol, 10 mM  $\beta$ -mercaptoethanol), and then stored at 4°C for kinetic experiments. Protein concentrations were determined from the extinction coefficient (102,400 M $^{-1}$  cm $^{-1}$ ) calculated from the sequences of HisZ and HisG, incorporating a weight average of the two subunit types, as described in (16).

### **ATP-PRT Assay**

The phosphoribosyl transferase activity of ATP-PRT was monitored at  $A_{200}$  as an increase in the formation of PR-ATP over time (22). The measurements were performed at 22 °C in 100 mM Tris HCl pH 8.5 containing 10 mM MgCl<sub>2</sub>, 150 mM KCl, 5 mM β-mercaptoethanol, and inorganic pyrophosphatase at 2 U/mL. Purified proteins were added to the concentration indicated, and the reactions were initiated by the addition of PRPP. The absorbance was detected every 9 sec for 10 min after initiation of the reaction. The baseline absorbance was established by zeroing the spectrophotometer before adding the PRPP. The extinction coefficient for PR-ATP is 3600 M<sup>-1</sup> cm<sup>-1</sup> (23), and was used to convert the absorbance units per minute to picomoles of product formed per second. Steady state parameters K<sub>m</sub>PRPP and  $K_m^{ATP}$  were determined by varying ATP or PRPP while holding the other substrate at a fixed concentration. The PRPP kinetics were measured using a range of 10 µM-1 mM in PRPP, with ATP at 5 mM. For some mutants, the highest concentration of PRPP had to be adjusted upward to accommodate poorer binding. Similarly, the variable ATP assays were performed using ATP concentration in the range of 100 µM to 5 mM, maintaining the PRPP concentration at 2 mM. The initial rates were calculated from the linear portions of the progress curves, and then the steady state parameters were obtained from saturation plots of V/[E<sub>0</sub>] versus [S]. Each substrate concentration was performed three to five times, such that the plots were derived using the mean value of V/[E<sub>o</sub>]. Turnover numbers (k<sub>cat</sub>) were calculated from the quotient of the maximal catalytic rate divided by the total concentration of active sites. The kinetic parameters for all mutant proteins (except S140A, which proved to be unstable) were measured as described above. The concentrations of the mutant enzymes were adjusted upward when necessary to achieve an initial rate of PR-ATP formation that was approximately equivalent to the wild type enzyme. Reactions that included inhibitors were prepared by adding AMP or histidine to the 2× reaction buffer, pre-incubated for five minutes in the presence of enzyme, and then initiated by the addition of PRPP. The AMP concentrations employed in the inhibitor assays spanned a range from 0-10 mM, while histidine concentrations ranged from 0-200 μM.

### Data fitting and analysis

The kinetic parameters including  $K_m$ ,  $V_{max}$  were extracted by directly fitting the initial rate versus substrate data to the Michaelis-Menten equation:

$$V = \frac{V_{\max}[S]}{K_m + [S]}$$

Equation 1

This equation was used for experiments involving fixed PRPP and variable ATP, as well as fixed ATP and variable PRPP. The data from the histidine and AMP inhibition experiments were plotted as 1/V versus 1/[S] for each of the four different concentrations of histidine and AMP. The slopes of these lines were used to determine the  $K_i$  for histidine and AMP by plotting the  $K_{m\;app}/V_{max}$  values against [histidine] or [AMP]. The slope of the resulting line is equivalent to  $K_m/K_i$  and the abscissa intercept equals the  $-K_i$  value for competitive inhibition systems:

 $K_{mapp} = \frac{K_m}{K_i} [I] + K_m$ 

Equation 2

### **RESULTS**

## Steady state kinetic parameters for the PRT reaction catalyzed by the hetero-octameric ATP-PRTase reaction

The reaction catalyzed by ATP-PRT, and a ribbon diagram of the structure of the L. lactis ATP-PRT hetero-octameric complex, are presented in Figure 1. As described in "Experimental Procedures", the steady state kinetic parameters were determined by use of a continuous assay in which the production of PR-ATP is monitored by the increase absorbance at 290 nm. Under conditions where the concentration of ATP was fixed at 5 mM and that of PRPP was varied, straightforward Michaelis-Menten behavior was observed, allowing kinetic parameters of  $k_{cat} = 2.67 \pm 0.27 \text{ s}^{-1}$  and  $K_m^{PRPP} = 18.4 \pm 3.5 \mu\text{M}$  to be readily determined (Figure 3A). The  $K_m$  for PRPP for the "short form" enzyme compares reasonably with the values of  $K_m^{\ PRPP}$ determined for "long form" ATP-PRTs (summarized in Table 1), which range from values of 11–67 μM determined for the S. typhimurium enzyme (1,20) to 130 μM determined for the enzyme from *Arabidopsis thalia* (24). This value for  $K_m^{PRPP}$  is also comparable to the corresponding parameters for other PRTs, including the orotate PRT ( $K_m$ = 18.7  $\mu$ M), quinolate PRT ( $K_m^{PRPP}=30~\mu M$ ) and the HPRTs from Tritirichomonas foetus ( $K_m=46~\mu M$ ) and Trypanosoma cruzi (32.4 μM). Thus, similar Michaelis constants for the PRPP substrate are observed across the entire PRTs superfamily. The level of catalytic activity of the short form ATP-PRT as determined in our assay is comparable to that measured for the "long form" enzyme from Salmonella measured earlier (20,25), but significantly slower than the HPRTs from the type I PRT families, which exhibited  $k_{cat}$  in the range of 23–76 s<sup>-1</sup>. These enzymes lack the complex regulation of ATP-PRT, and are invariably dimers. Kinetic parameters were also determined under conditions where PRPP was the constant substrate at 2 mM, and ATP was the variable substrate. Over a concentration range of 100 μM to 5 mM in ATP, reliable fits to the Michaelis-Menten equation were obtained, providing kinetic parameters of k<sub>cat</sub> =  $2.3 \pm 0.25~sec^{-1}$ , and  $K_m^{ATP} = 2.4 \pm 0.24~mM$  (Figure 3B). Notably, this value is significantly higher than the values of  $K_m^{ATP}$  reported for other ATP-PRTs. For example, the  $K_m^{ATP}$  for the S. typhimurium enzyme was reported as 110-200 μM (1,20), while values of 600 μM and 510 µM were determined the two isozymes of A. thalia (24). Non-physiological concentrations of ATP in excess of 15 mM were inhibitory to ATP-PRT, which may reflect a competition by ATP for the PRPP binding site, as proposed for AMP (14). This phenomenon remains to be investigated in more depth.

### Kinetic parameters of inhibition by histidine and AMP

The inhibition of the *L. lactis* enzyme by histidine was determined from velocity versus PRPP concentration plots at a constant concentration of ATP at 5 mM, and over histidine concentrations ranging from 0 to 200  $\mu$ M (Figure 4A). Replot of the slopes of the reciprocal plots against histidine concentration returned a value of 81  $\mu$ M, indicating that, to a first

approximation, histidine is a non-competitive inhibitor (Figure 4B). This value is in good agreement with early published values (70–100 μM) for the S. typhimurium enzyme, but there was an early discrepancy in the literature about whether this inhibition is non-competitive or uncompetitive (1, 26). Vega et al. (18) reported that histidine is a non-competitive inhibitor of the hetero-octameric *Thermotoga maritima* enzyme with a  $K_i$  of 350  $\pm$  20  $\mu$ M, while Ohta et al. reported IC<sub>50</sub>'s for inhibition by histidine of the A. thalia ATP-PRT1 and ATP-PRT2 of 45 and 320 µM, respectively (24). Non-competitive inhibition by histidine with respect to PRPP for the short form of HisG is consistent with prior data suggesting that the histidine binding site is located in the HisZ subunit, at least 43 Å from the C1' on PRPP where chemistry occurs (19). It should be noted that reciprocal plots in Figure 4A appear to intersect below the horizontal axis, which at least formally raises the possibility that the ESI complex has at least a fraction of the activity of the ES complex (27). A similar experimental strategy was used to determine the K<sub>i</sub> for AMP with PRPP. As determined from the replot in Figure 4D, the K<sub>i</sub> for AMP is 1.81 mM, and represents competitive inhibition with respect to PRPP. This value is within a factor of three of the reported  $K_i$  for AMP (5 mM) for the enzyme from S. typhimurium (1).

### Mutations in the PRPP binding site result in a loss of catalytic activity

The similarities in steady state kinetic parameters between the hetero-octameric and hexameric ATP-PRTs, as well as their relationship to the parameters for the more distantly related type I PRTs, motivated a more detailed analysis of the contacts to PRPP suggested by the L. lactis crystal structure (Figure 5). The three moieties of PRPP that are directly recognized by the enzyme include the 5' phosphate, the 2' and 3' ribose hydroxyl groups, and the pyrophosphate group (19). In both the long and short form enzymes, the 5'phosphate is recognized by side chains in HisG  $\beta$ 9 and  $\alpha$ 7, including the main chain amides at T159 and G160, and the  $\gamma$ OH groups of T159 and T162. The two latter residues are highly conserved in ATP-PRTs, the only exceptions being the substitution of T159 with serine in the hexameric enzymes from eukaryotes (Figure 2). Pyrophosphate recognition appears to be provided by basic and polar side chains contributed from diverse structural elements, including the  $\beta 1/\alpha 1$  loop, the  $\beta 3/\alpha 2$ loop, and the N-terminus of  $\alpha 6$  (Figure 2). To explore the contributions of these contacts to catalysis in detail, six of the most conserved contacts, including K8, K50, S140, D155, D159, and T162 were substituted with alanine. The resulting mutant proteins were then characterized with respect to their steady state parameters. As indicated in Figure 6 and Table 2, the substitutions that proved to be the most deleterious were those associated with interactions to the 5' phosphate. The T159A and T162A mutants were both significantly compromised for PRPP binding, with 279-fold and 49-fold increases in K<sub>m</sub>PRPP (Figure 6 A,B). In contrast, both mutants were much less affected at the level of  $k_{cat}$  (only 2–3 fold decreased relative to wild type) and exhibited slightly decreased values for  $K_m^{ATP}$  (Figure 6 C,D). These latter parameters indicate that the effect on PRPP binding is likely to be a consequence of the loss of a specific contact, and not the result of a global structural defect. The D155A mutant substitutes a carboxylate contact to the 2' OH of PRPP that is nearly invariant in both the HisG<sub>L</sub> and HisG<sub>S</sub> ATP-PRT subfamilies. Of all mutants tested in the study, D155A was the mutant least affected with respect to enzymatic activity, exhibiting no effect on k<sub>cat</sub>, and only 3.8- and 1.7fold increases in the Michaelis constants for PRPP and ATP, respectively (Table 2). Thus, this contact appears to provide only a modest (< 0.5 kcal mol<sup>-1</sup>) stabilization of PRPP binding.

The two side chains in the *L. lactis* complex best positioned to interact with the pyrophosphate group include S140 and K8'. Notably, these are implicated in conformational changes associated with the activation process (19). S140 is located in  $\alpha$ 6, which undergoes a coil to helix transition upon activation, while K8' makes a relatively long range (~3 Å) cross-subunit hydrogen bond to the  $\beta$  phosphate of the PPi moiety that is specific to the activated state. A third possible candidate for pyrophosphate interaction is K50' which, like K8', also reaches

across the dimmer interface. Its functional role is less clear from the structure, because it can also make potential interactions with  $\beta$  and  $\gamma$  groups of ATP (19). All three residues are highly conserved in both the hexameric and hetero-octameric ATP-PRT families, but K8 and K50 are substituted with serine in some taxa, and S140 is frequently substituted with alanine in the archaeal and eukaryotic versions of HisGL (Figure 2). In the *L. lactis* enzyme, alanine substitutions of K8' and K50' produced stable enzymes that could be readily characterized. The K8A mutant had the milder effect, with  $k_{cat}$  decreased by a modest 2.5-fold,  $K_m^{ATP}$  increased 3.5-fold, and  $K_m^{PRPP}$  increased 4.5-fold (Table 2). The K50A substitution produced a more substantial effect, causing  $K_m^{PRPP}$  and  $K_m^{ATP}$  to increase 47-fold and two-fold, respectively. Unexpectedly, the overall  $k_{cat}$  for the reaction increased 2.5-fold. Thus, while neither side chain could be said to be essential for catalysis, mutations at both diminished ground state binding to both PRPP and ATP.

All of the aforementioned mutants could be readily expressed and purified, suggesting an absence of obvious effects on protein stability. In contrast, the introduction of the S140A substitution into the L lactis enzyme dramatically decreased expression of  $HisG_S$  to the extent that experimentally useful amounts of S140A hetero-octamer could not be obtained. Attempts to detect any PRT activity in the residual amount of complex that could be captured by the Ni-NTA column were unsuccessful, and the subunit stoichiometry of the eluted material could not be determined. Accumulation of the regulatory HisZ subunit did not seem to be affected by the mutation, suggesting that each subunit is not strictly dependent on the other to preserve stability. At a minimum, these results suggest that S140 plays an essential role in the either the folding of  $HisG_S$ , or serves to stabilize the quaternary structure of the octamer. From these results alone, the potential role of S140 in catalysis could not be assessed.

### Coupling of histidine mediated inhibition to substrate binding by ATP PRT

In our published model to account for the effect of histidine on ATP-PRT function, the binding of histidine to a site on the HisZ subunit causes a rearrangement of the HisZ-HisG<sub>S</sub> interface, which is ultimately relayed through secondary structure elements to the side chains that directly contact PRPP (19). As a first test of this model, we compared the sensitivity of two different mutants, D155A and K50A, to the presence of 75 µM histidine, a concentration nearly equivalent to  $K_i$  for the wild type enzyme. These two side chains were selected to provide a comparison between a residue with a predicted role in allosteric transmission to one with no obvious connection to the predicted structural pathway for histidine inhibition. For the former, K50A is located at the amino terminus of His $G_S \alpha 2$ , an  $\alpha$ -helix whose C-terminal end is located in the HisG<sub>S</sub> dimeric interface that abuts HisZ. In our model, histidine binding to HisZ is expected to transmit structural changes to the ATP-PRT binding site via the HisZ-HisG interface and α2. In contrast, D155 is a highly conserved residue on β9 that contacts the 2'OH of PRPP, but is not connected to a secondary structure element that undergoes conformational change upon histidine binding. In the presence of 75 µM histidine, the kinetic parameters for D155A and K50A were both altered, indicating that the effect of histidine binding has not been completely uncoupled in either mutant (Table 2). The magnitudes of the perturbations were, however, significantly different for the two mutants. Histidine at a concentration of 75  $\mu M$  had a comparatively modest effect on D155A, as  $k_{cat}$  and  $K_m^{\ PRPP}$  were decreased by factors of only 1.6- and 1.9-fold, respectively (Figure 7A). In contrast, the k<sub>cat</sub> parameter was decreased 6.8-fold, and K<sub>m</sub><sup>PRPP</sup> was decreased 25.8-fold for the K50A mutant (Figure 7B). For both mutants, therefore, the presence of histidine increased the affinity of enzyme for PRPP, and decreased overall k<sub>cat</sub>. The comparatively greater change in parameters for the K50A mutant is consistent with its proposed involvement in the transduction of the allosteric signal associated with histidine binding. However, the enhancement of PRPP binding seen in the presence of histidine for K50A provides some of the first direct kinetic evidence of the structural

relationship between substrate and inhibitor binding, and underscores the complex nature of regulation in this system.

### DISCUSSION

# The biological basis for ATP-PRT kinetic parameters: optimization in response to intracellular substrate and inhibitor concentrations

The original studies on ATP-PRT were motivated by an appreciation of its importance in biosynthetic regulation. These studies estimated the energetic cost of histidine biosynthesis in *S. typhimurium* to be on the order of ~41 ATPs, representing a substantial physiological burden (28). More recently, a genome scale modeling analysis of total *E. coli* physiology highlighted the ATP-PRT reaction as one of a limited number of key biosynthetic reactions that are thermodynamically unfavorable yet essential for bacterial growth (29). These empirical and theoretical observations illustrate why selective pressures may have led to complex regulatory features in ATP-PRT that allow it to control histidine biosynthesis at the metabolic level. The principal goal of this study was to explore the functional differences of the two distinct forms of ATP-PRT, both of which appear to share a common evolutionary ancestry (12), yet possess different quaternary structures (13,14,18,19). Identification of functional features that separate the two classes should help to account for their persistence in contemporary organisms.

A comparison of the parameters for the hetero-octameric enzyme with those for the hexameric enzymes indicates that, on balance, similarities outweighed differences. The value of  $K_m^{PRPP}$  determined for the L. lactis enzyme (18.4 ± 3.5  $\mu$ M) is similar to the values determined for the hexameric prokaryotic enzymes, but lower than the values for the two isoforms of A. thalia (Table 1). Notably, the Michaelis constants for PRPP for the two ATP-PRT classes in prokaryotic enzymes are significantly less than the estimated intracellular concentration of PRPP in S. typhimurium, which has been measured at 350 µM (30). Under typical physiological conditions, therefore, the hexameric and hetero-octameric forms of the enzyme would both be expected to be saturated with PRPP, providing little regulation by this substrate. However, the intracellular PRPP pool is likely to vary in a complex fashion in response to the summed contribution of the fluxes through the histidine and purine biosynthesis pathways (31). In addition to sharing PRPP as a common precursor, both pathways share 5'-phosphoribosyl-4carboxyamide-5 aminoimidazole (AICAR) as a common intermediate, providing a direct basis for additional integration. It is therefore not surprising that the K<sub>m</sub>PRPP reported here for the ATP-PRTs is similar in magnitude to the Michaelis constants for PRPP determined for other PRTs in nucleotide biosynthesis and salvage pathways (32), including OPRT (33), OPRT (34), and HGPRT (32). Thus, the size of the PRPP pool is likely to regulate histidine biosynthesis by virtue of the competition between ATP-PRT and the other enzymes in the nucleotide and amino acid biosynthetic pathways that employ PRPP as substrate.

The long and short forms similarly exhibit nearly identical non-competitive  $K_i$ 's for histidine, in the range of 70–100  $\mu$ M. This value lies between the intracellular concentration of histidine in cells growing on media lacking histidine (25  $\mu$ M), and cells growing in the presence of 50  $\mu$ g/mL (100  $\mu$ M), suggesting that both forms of ATP-PRT are "tuned" to be maximally responsive to changes in histidine concentration over a relatively tight but biological significant concentration range (3). Not surprisingly, the value of  $K_i^{His}$  determined here is nearly equal to the histidine Michaelis constant for histidyl-tRNA synthetase ( $K_m^{His}$  =35  $\mu$ M), which possesses a structurally similar binding pocket (35). In view of the structural differences between the histidine binding domains of the two enzyme families (13,14,18,19), this nearly identical value of the  $K_i$  for histidine for the long and short forms is striking. The higher  $K_i^{His}$  (350  $\mu$ M) reported for the *T. maritima* enzyme (18) represents a significant outlier from these values, but could be a consequence of performing the measurement at 20 °C, below the physiologically optimal temperature for *Thermatoga*, which is 80 °C It is notable that the two

isoforms of ATP-PRT in A. thalia, which may be located in different intracellular compartments, differ significantly with respect to their  $IC_{50}$ 's for histidine (24). Histidine biosynthesis for this organism may be under different control in the cytoplasm and chloroplast, but definitive proof that ATP-PRT controls histidine flux in plants has not yet been reported.

While the parameters for PRPP and histidine were essentially identical between the two ATP-PRT families, K<sub>m</sub><sup>ATP</sup> differed by a factor of more than ten (Table 1). The K<sub>m</sub><sup>ATP</sup> for the long form enzyme is significantly below the 3 mM intracellular concentration of ATP (36), while that of the short form enzyme is approximately equal to it. Early work by Atkinson and coworkers showed that the regulation of the hexameric ATP-PRT by energy charge is sensitized in the presence of histidine (37,38), consistent with the synergistic binding effects of histidine and the competitive inhibitor AMP indicated from kinetic and structural analysis. The higher value of K<sub>m</sub><sup>ATP</sup> for the hetero-octameric enzyme could reflect a requirement for tighter regulation of ATP-PRT by energy charge in the absence of histidine, a possibility that remains to be addressed in future work. Intriguingly, the presence of histidine increased k<sub>cat</sub>/K<sub>m</sub>PRPP for K50A to within a factor of five relative to the wild type (Table 2, Figure 7). This suggests that the K50A mutation unmasks the structural linkage between substrate and histidine binding, perhaps as a consequence of the direct role of this residue in the allosteric pathway. Preliminary results from an investigation of HisZ-HisG interface mutants (Piscitelli, E. and C.S.F., unpublished) reveals similar behavior, underscoring the complex relationship between substrate and inhibitor binding.

### Implications for the mechanism of the ATP-PRT reaction

The principal points of contact on PRPP for ATP-PR active site side chains include the 5' phosphate, the 2' and 3' ribose hydroxyls, and the pyrophosphate group (Figure 5). In our published model for the activation of the ATP-PRT reaction, secondary structure elements responsible for recognition of the 5' phosphate and 2',3' ribose hydroxyls of PRPP undergo minimal conformational change in response to substrate binding, while those that interact with the pyrophosphate group must be mobilized from sequestered locations in the structure during the activation process (19). In particular, the highly conserved PRPP loop does not undergo any structural changes during the activation process, such that its interactions with PRPP would be expected to provide relatively uniform binding across the reaction coordinate. Consistent with this prediction, we found that T159A and T162A increased  $K_{\rm m}^{\rm PRPP}$ , but had relatively minor effects on  $k_{\rm cat}$  (Table 2). The D155A mutant had a qualitatively similar, but smaller, effect. Contacts with the 5' phosphate and the 2'OH are therefore likely to represent essentially ground state interactions. This is consistent with the competitive nature of AMP binding with respect to both PRPP and ATP, which appears to be stabilized by interactions between the 5' phosphate and the conserved PRPP loop (14).

Early kinetic isotope work indicated that the transition state for ATP-PRT has significant dissociative character, suggesting that the bond to the leaving group is lengthened considerably before appreciable bond formation to the nucleophile has occurred (21). Here, we examined the potential role of leaving group stabilization by mutating K8, K50, and S140, the basic and or polar side chains that represent the most likely candidates for interactions with the pyrophosphate moiety upon activation. None of these, however, decreased  $k_{cat}$  to a significant extent, arguing against the idea that neutralizing interactions between these basic side chains and the negatively charged pyrophosphate group strengthen as the reaction approaches the transition state. (The role of interactions by S140 could not be assessed owing to the instability of the resulting mutant protein.) However, K8A and K50A increased  $K_{m}^{PRPP}$ , and K50A was in fact associated with a modest increase in  $k_{cat}$ . If K50A is important for uniform binding to PRPP and ATP over the entire reaction coordinate, a weaker interaction with the PR-ATP product might be expected to accelerate the rate of product release. Similar observations were

made with regard to the effects of the K68E mutant in T. cruzii HPRT on  $K_m$  PRPP and  $k_{cat}$  (39). Like K8 and K50 in ATP-PRT, K68 is implicated in pyrophosphate binding in HPRT. In both systems, therefore, interactions to the pyrophosphate moiety may therefore be contributing uniform binding free energy to catalysis, as opposed to differential stabilization to the transition state (40). These observations argue against the idea that chemistry is rate limiting for the overall reaction, but are consistent with the general observation that, for many PRTs, product release is the rate determining step (6).

Detailed analysis of the transition states for nucleoside phosphorylase (41) and OPRT (42) suggest that an important feature of the PRT mechanism is the concept of "nucleophillic displacement" (6). For nucleoside phosphorylase, this involves relatively fixed interactions to the nucleobase leaving group and Pi nucleophile, but a clear migration of C1' (on the order 3 Å) from the leaving group to the Pi nucleophile. For both of these enzymes, a significant number of the electrostatic interactions that serve to stabilize the transition state are intramolecular, and are not provided by direct interactions with active site chains. For example, the developing positive charge on O4' in the ribooxocarbenium ion is stabilized by negative charges contributed from the 5' phosphate and Mg<sup>2+</sup>: pyrophosphate moieties. Achieving this stabilization requires a specific geometry in the substrate that is enforced by the enzyme active site, which also prevents solvent water molecules from the attacking reactive species like the oxocarbenium ion. If this view is a reasonable picture of the ATP-PRT mechanism, than the principle contribution the enzyme makes to enhance the rate of catalysis is to limit conformational and translational freedom in the substrates, thereby lowering the free energy of activation. Such a view could explain the absence of obvious "catalytic" residues in ATP-PRT whose substitution would be expected to diminish k<sub>cat</sub> by significant factors greater than two to three orders of magnitude (43).

While ATP-PRTs and type I PRTs are defined by structurally unrelated catalytic domains, functionally homologous active site loops and secondary structure elements can be identified that appear to underlie similarities in mechanism. Thus, the PRPP loop of type I PRTs, with its characteristic pair of acidic residues and conserved TXXT motif can be readily superimposed (r.m.s.d. = 0.5-0.8Å) on the PRPP loop of ATP PRTs (14). Recognition of the pyrophosphate group by type I PRTs is mediated by two elements, the "PPi loop" found between  $\beta 1$  and  $\alpha 2$  that exhibits minimal sequence conservation (save for a rare non-proline cis peptide between the first and second residues of the loop), and the "flexible loop", a conformationally mobile structural element that is only ordered when Mg<sup>2+</sup>-PRPP is bound in the active site. Notably, mutagenesis studies on type I PRTs focusing on the PPi and flexible loops have been reported, and indicate that, similar to our results with K8A and K50A, substitutions of PRPP contacting residues preferentially increase K<sub>m</sub>PRPP without imposing significant effects on k<sub>cat</sub> (39,44). In a study that correlated mutations in human HPRT with various in vitro and in vivo phenotypes, among the recurring mutations were substitutions that affected residues responsible for binding Mg<sup>2+</sup>, which is coordinated to the pyrophosphate (45). One perplexing issue not yet addressed in any of the ATP-PRT structure studies or the work reported here is the absence of information concerning Mg<sup>2+</sup> binding. Divalent metal is required for activity, but there is no structural evidence for it in any of the complexes reported thus far. As yet, none of the available ATP-PRT crystal structures has provided information about the pattern of Mg<sup>2+</sup> binding in the type IV enzyme, so similarities in metal binding cannot be assessed. This important question remains to be addressed in future studies.

### Acknowledgements

The authors thank Ethan Guth and Robert Hondal for useful discussions and comments on the manuscript, and Tim Hunter of the Vermont Cancer Center DNA Sequencing Facility for DNA analysis services.

### **ABBREVIATIONS**

**AICAR** 

5'-phosphoribosyl-4-carboxyamide-5 aminoimidazole

ATP-PRT

ATP phosphoribosyltransferase

**PRT** 

phosphoribosyltransferase

**HisRS** 

histidyl-tRNA synthetase

**HPRT** 

hypoxanthine-guanine phosphoribosyltransferase

**OPRT** 

orotidine phosphoribosyltransferase

**QPRT** 

quinolate phosphoribosyltransferase

**PRPP** 

5-phosphoribosyl-1-pyrophosphate

PR-ATP

N-1-(5'-phosphoribosyl)-ATP

### References

 Martin RG. the first enzyme in histidine biosynthesis: the nature of feedback inhibition by histidine. J Biol Chem 1963;238

- 2. Morton DP, Parsons SM. Inhibition of ATP phosphoribosyltransferase by AMP and ADP in the absence and presence of histidine. Arch Biochem Biophys 1977;181:643–8. [PubMed: 332083]
- 3. Winkler, ME. Biosynthesis of Histidine. Escherichia coli and Salmonella Typhimurium. In: Neidhardt, FC., editor. Cellular and Molecular Biology. American Society for Microbiology; Washington, D. C.: 1987. p. 395-411.
- 4. Ames BN, Martin RG, Garry BJ. The first step of histidine biosynthesis. J Biol Chem 1961;236:2019–26. [PubMed: 13682989]
- 5. Blasi F. Gene expression of the histidine operon. Acta Microbiol Acad Sci Hung 1976;23:151–9. [PubMed: 788469]
- Schramm VL, Grubmeyer C. Phosphoribosyltransferase mechanisms and roles in nucleic acid metabolism. Prog Nucleic Acid Res Mol Biol 2004;78:261–304. [PubMed: 15210333]
- 7. Musick WD. Structural features of the phosphoribosyltransferases and their relationship to the human deficiency disorders of purine and pyrimidine metabolism. CRC Crit Rev Biochem 1981;11:1–34. [PubMed: 7030616]
- 8. Smith JL. Forming and inhibiting PRT active sites. Nat Struct Biol 1999;6:502-4. [PubMed: 10360346]
- 9. Sinha SC, Smith JL. The PRT protein family. Curr Opin Struct Biol 2001;11:733–9. [PubMed: 11751055]
- 10. Kim C, Xuong NH, Edwards S, Madhusudan Yee MC, Spraggon G, Mills SE. The crystal structure of anthranilate phosphoribosyltransferase from the enterobacterium Pectobacterium carotovorum. FEBS Lett 2002;523:239–46. [PubMed: 12123839]
- 11. Sharma V, Grubmeyer C, Sacchettini JC. Crystal structure of quinolinic acid phosphoribosyltransferase from Mmycobacterium tuberculosis: a potential TB drug target. Structure 1998;6:1587–99. [PubMed: 9862811]

12. Bond JP, Francklyn C. Proteobacterial histidine-biosynthetic pathways are paraphyletic. J Mol Evol 2000;50:339–47. [PubMed: 10795825]

- 13. Cho Y, Sharma V, Sacchettini JC. Crystal structure of ATP phosphoribosyltransferase from Mycobacterium tuberculosis. J Biol Chem 2003;278:8333–9. [PubMed: 12511575]
- 14. Lohkamp B, McDermott G, Campbell SA, Coggins JR, Lapthorn AJ. The structure of Escherichia coli ATP-phosphoribosyltransferase: identification of substrate binding sites and mode of AMP inhibition. J Mol Biol 2004;336:131–44. [PubMed: 14741209]
- 15. Wang Z, Luecke H, Yao N, Quiocho FA. A low energy short hydrogen bond in very high resolution structures of protein receptor--phosphate complexes. Nat Struct Biol 1997;4:519–22. [PubMed: 9228942]
- Bovee ML, Champagne KS, Demeler B, Francklyn CS. The quaternary structure of the HisZ-HisG N-1-(5'-phosphoribosyl)-ATP transferase from Lactococcus lactis. Biochemistry 2002;41:11838– 46. [PubMed: 12269828]
- 17. Sissler M, Delorme C, Bond J, Ehrlich SD, Renault P, Francklyn C. An aminoacyl-tRNA synthetase paralog with a catalytic role in histidine biosynthesis. Proc Natl Acad Sci U S A 1999;96:8985–90. [PubMed: 10430882]
- Vega MC, Zou P, Fernandez FJ, Murphy GE, Sterner R, Popov A, Wilmanns M. Regulation of the hetero-octameric ATP phosphoribosyl transferase complex from Thermotoga maritima by a tRNA synthetase-like subunit. Mol Microbiol 2005;55:675–86. [PubMed: 15660995]
- 19. Champagne KS, Sissler M, Larrabee Y, Doublie S, Francklyn CS. Activation of the hetero-octameric ATP phosphoribosyl transferase through subunit interface rearrangement by a tRNA synthetase paralog. J Biol Chem 2005;280:34096–104. [PubMed: 16051603]
- 20. Morton DP, Parsons SM. Biosynthetic direction substrate kinetics and product inhibition studies on the first enzyme of histidine biosynthesis, adenosine triphosphate phosphoribosyltransferase. Arch Biochem Biophys 1976;175:677–86. [PubMed: 183121]
- 21. Goitein RK, Chelsky D, Parsons SM. Primary 14C and alpha secondary 3H substrate kinetic isotope effects for some phosphoribosyltransferases. J Biol Chem 1978;253:2963–71. [PubMed: 641051]
- 22. Voll MJ, Appella E, Martin RG. Purification and composition studies of phosphoribosyladenosine triphosphate:pyrophosphate phosphoribosyltransferase, the first enzyme of histidine biosynthesis. J Biol Chem 1967;242:1760–7. [PubMed: 5337591]
- 23. Ames BN, Hartman PE, Jacob F. Chromosomal alterations affecting the regulation of histidine biosynthetic enzymes in Salmonella. J Mol Biol 1963;7
- 24. Ohta D, Fujimori K, Mizutani M, Nakayama Y, Kunpaisal-Hashimoto R, Munzer S, Kozaki A. Molecular cloning and characterization of ATP-phosphoribosyl transferase from Arabidopsis, a key enzyme in the histidine biosynthetic pathway. Plant Physiol 2000;122:907–14. [PubMed: 10712555]
- 25. Kleeman JE, Parsons SM. Reverse direction substrate kinetics and inhibition studies on the first enzyme of histidine biosynthesis, adenosine triphosphate phosphoribosyltransferase. Arch Biochem Biophys 1976;175:687–93. [PubMed: 183122]
- 26. Whitfield HJ Jr. Purification and properties of the wild type and a feedback-resistant phosphoribosyladenosine triphosphate pyrophosphate phosphoribosyltransferase, the first enzyme of histidine biosynthesis in Salmonella typhimurium. J Biol Chem 1971;246:899–908. [PubMed: 5543688]
- Segel, I. Enzyme Kinetics: beahvior and analysis of rapid equilibrium and steady state kinetics. Wiley; New York: 1975.
- 28. Brenner, M.; Ames, BN. The histidine operon and its regulation. In: Voge, HJ., editor. Metabolic Regulation. Academic Press; New York: 1971.
- 29. Hatzimanikatis V, Li C, Ionita JA, Henry CS, Jankowski MD, Broadbelt LJ. Exploring the diversity of complex metabolic networks. Bioinformatics 2005;21:1603–9. [PubMed: 15613400]
- 30. Sadler WC, Switzer RL. Regulation of Salmonella phosphoribosylpyrophosphate synthetase activity in vivo. Deductions from pool measurements. J Biol Chem 1977;252:8504–11. [PubMed: 200613]
- 31. Pendyala L, Wellman AM. Effect of histidine on purine nucleotide synthesis and utilization in Neurospora crassa. J Bacteriol 1975;124:78–85. [PubMed: 126229]

32. Munagala N, Basus VJ, Wang CC. Role of the flexible loop of hypoxanthine-guanine-xanthine phosphoribosyltransferase from Tritrichomonas foetus in enzyme catalysis. Biochemistry 2001;40:4303–11. [PubMed: 11284686]

- 33. Wang GP, Lundegaard C, Jensen KF, Grubmeyer C. Kinetic mechanism of OMP synthase: a slow physical step following group transfer limits catalytic rate. Biochemistry 1999;38:275–83. [PubMed: 9890908]
- 34. Cao H, Pietrak BL, Grubmeyer C. Quinolinate phosphoribosyltransferase: kinetic mechanism for a type II PRTase. Biochemistry 2002;41:3520–8. [PubMed: 11876660]
- 35. Francklyn C, Adams J, Augustine J. Catalytic Defects in Mutants of Class II Histidyl-tRNA Synthetase from *Salmonella typhimurium* Previously Linked to Decreased Control of Histidine Biosynthesis Regulation. J Mol Biol 1998;280:847–858. [PubMed: 9671554]
- 36. Neuhard, J.; Nygaard, P. Biosynthesis and Conversions of Nucleotides. 1. American Society for Microbiology; Washington, D. C.: 1987.
- 37. Klungsoyr L, Atkinson DE. Regulatory properties of phosphoribosyladenosine triphosphate synthetase. Synergism between adenosine monophosphate, phosphoribosyladenosine triphosphate, and histidine. Biochemistry 1970;9:2021–7. [PubMed: 4909873]
- 38. Klungsoyr L, Hagemen JH, Fall L, Atkinson DE. Interaction between energy charge and product feedback in the regulation of biosynthetic enzymes. Aspartokinase, phosphoribosyladenosine triphosphate synthetase, and phosphoribosyl pyrophosphate synthetase. Biochemistry 1968;7:4035–40. [PubMed: 4881060]
- 39. Canyuk B, Medrano FJ, Wenck MA, Focia PJ, Eakin AE, Craig SP 3rd. Interactions at the dimer interface influence the relative efficiencies for purine nucleotide synthesis and pyrophosphorolysis in a phosphoribosyltransferase. J Mol Biol 2004;335:905–21. [PubMed: 14698288]
- Albery WJ, Knowles JR. Evolution of enzyme function and the development of catalytic efficiency. Biochemistry 1976;15:5631–40. [PubMed: 999839]
- 41. Fedorov A, Shi W, Kicska G, Fedorov E, Tyler PC, Furneaux RH, Hanson JC, Gainsford GJ, Larese JZ, Schramm VL, Almo SC. Transition state structure of purine nucleoside phosphorylase and principles of atomic motion in enzymatic catalysis. Biochemistry 2001;40:853–60. [PubMed: 11170405]
- 42. Tao W, Grubmeyer C, Blanchard JS. Transition state structure of Salmonella typhimurium orotate phosphoribosyltransferase. Biochemistry 1996;35:14–21. [PubMed: 8555167]
- 43. Kraut DA, Carroll KS, Herschlag D. Challenges in enzyme mechanism and energetics. Annu Rev Biochem 2003;72:517–71. [PubMed: 12704087]
- 44. Page JP, Munagala NR, Wang CC. Point mutations in the guanine phosphoribosyltransferase from Giardia lamblia modulate pyrophosphate binding and enzyme catalysis. Eur J Biochem 1999;259:565–71. [PubMed: 10092838]
- 45. Duan J, Nilsson L, Lambert B. Structural and functional analysis of mutations at the human hypoxanthine phosphoribosyl transferase (HPRT1) locus. Hum Mutat 2004;23:599–611. [PubMed: 15146465]

A.

B.

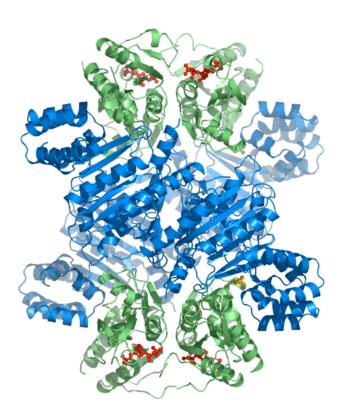


Figure 1. The ATP phosphoribosyltransferase reaction catalyzed by the hetero-octameric ATP PRT  $\,$ 

A., Reaction scheme illustrating the nucleophilic attack of N1 from ATP on C1' of PRPP. B., Ribbon diagram of the PRPP-bound complex of the hetero-octameric HisZ-HisG PRT from *Lactococcus lactis* (19). The four HisGS subunits are colored green, whereas the HisZ subunits are cyan. PRPP and inorganic phosphate are rendered in ball and stick mode in red and yellow, respectively.

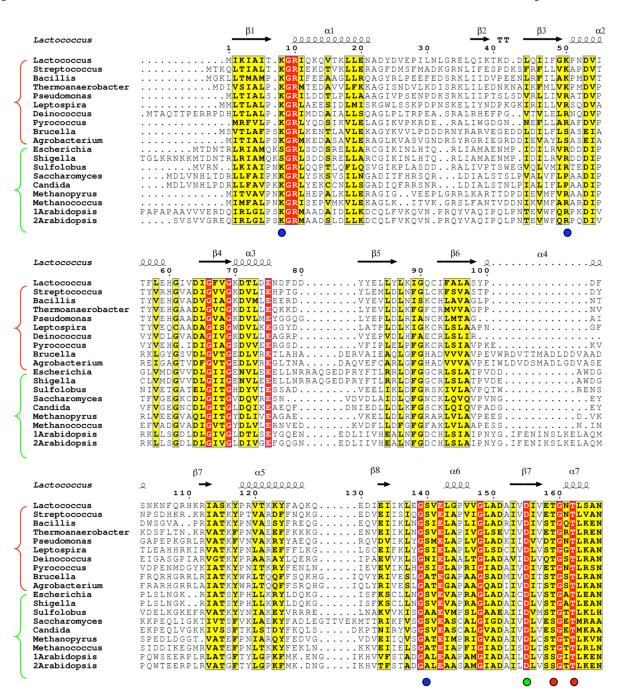
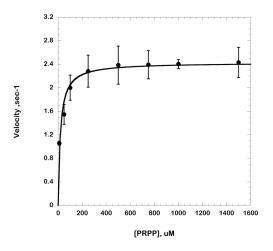


Figure 2. Multiple sequence alignment of representative HisGs and HisGL sequences, overlaid with the secondary structure assignments from L. lactis HisGs

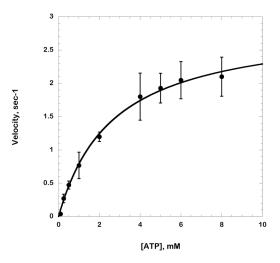
Sequences corresponding to HisG<sub>S</sub> are denoted in red brackets, and sequences corresponding to the HisG<sub>L</sub> form are denoted by green brackets. The alignment omits the histidine binding domain of HisG<sub>I</sub>, which is absent from HisG<sub>S</sub>. Blue circles beneath the alignment indicate residues in contact with the pyrophosphate moiety of PRPP, consisting of K8, K50, and S140. The green circle denotes D155. The T159 and T162 side chains are indicated by red circles. The organisms included in the alignment are: Lactococcus, Lactococcus lactis; Streptococcus, Streptococcus mutans; Thermoanerobacter, Thermoanerobacter tengcongenis;

Deinococcus radiodurans; Pyrococcus, Pyrococcus furiosis; Escherichia, Escherichia coli; Shigella, Shigella flexneri; Sulfolobus, Sulfolobus solfataricus; Saccharomyces, Saccharomyces cerevisiae; Candida, Candida albicans; Methanopyrus, Methanopyrus kandleri; Methanococcus; Methanococcus jannaschii; Brucella, Brucella melitensis; Agrobacterium, Agrobacterium tumefaciens; Arabidopsis, Arabidopsis thaliana (there are two isozymes i A. thaliana.)

A.

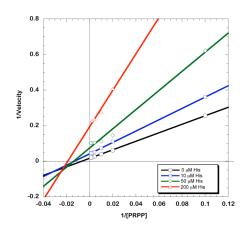


B.

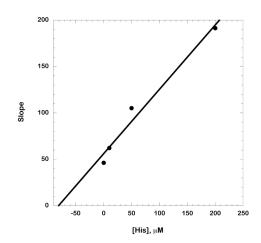


**Figure 3. Steady state kinetics for wild type heterooctameric ATP-PRT at 22** °C and pH 8.5 A., Plot of velocity versus varying concentration of PRPP, determined at constant (5 mM) ATP. Wild type ATP-PRT was added to a final concentration of 24.9 nM. B., Plot of velocity versus varying ATP concentration, determined at constant (2mM) PRPP. Wild type ATP-PRT was added to a final concentration of 27.3 nM. The experiments were performed as described in "Experimental Procedures." The error bars represent the standard error of the mean for the velocity measurements, which were measured three to five times. The 10–15% errors in the individual measurements were typical for the kinetics of the wild type and all of the mutants reported here.

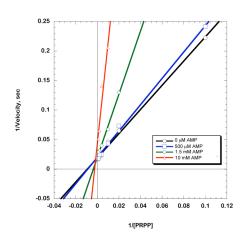
Α.



B.



C. D.



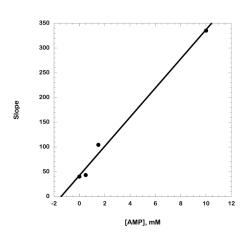
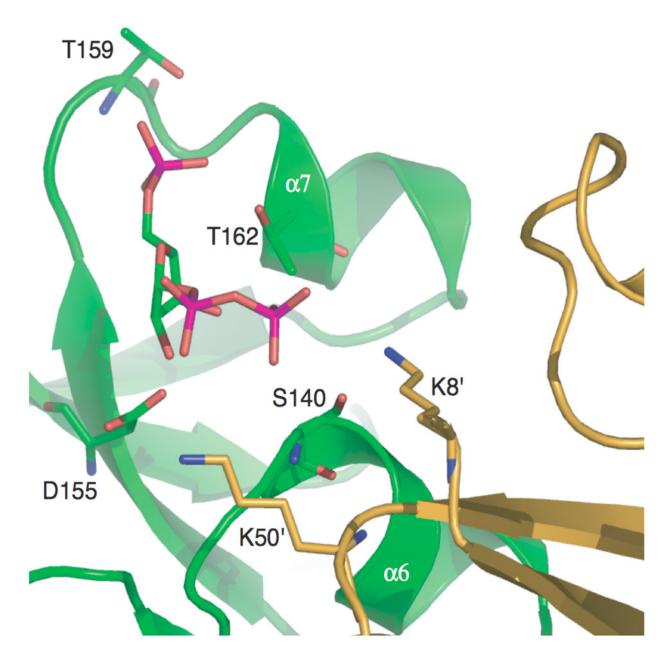


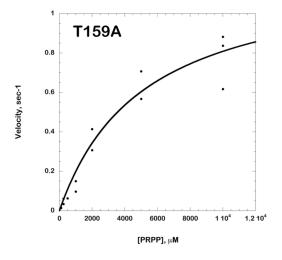
Figure 4. Inhibition ATP-PRT by histidine and AMP

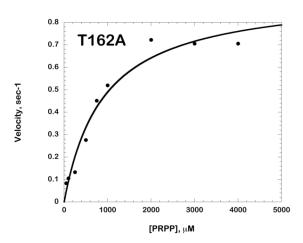
A., Lineweaver-Burke plots at a range of histidine concentrations from 0– 200  $\mu$ M. B., Replot of the slopes from plot A., against varying histidine. Wild type ATP-PRT was added to a final concentration of 39 nM. C., Lineweaver-Burke plots at a range of AMP concentrations from 0– 10 mM. D., Replot of the slopes from plot C., against varying AMP. Wild type ATP-PRT was added to a final concentration of 19.5 nM.



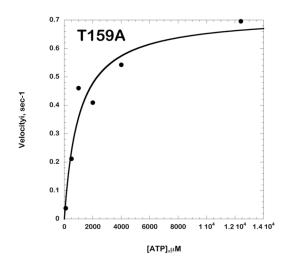
**Figure 5.** The PRPP binding site in ATP-PRT from *Lactococcus lactis*The hetero-octameric ATP-PRT active site from the *L. lactis* ATP-PRT complex (19) is rendered in ribbon representation, and contacts between PRPP and the enzyme mutated in this study are indicated. The bound substrate is rendered in stick representation, as are the conserved side chains substituted with alanine in this study.

A. B.





C. D.



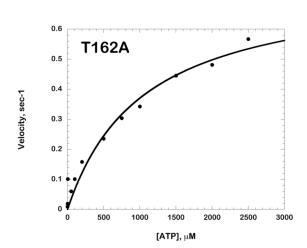


Figure 6. Steady state kinetics of PRPP loop mutants T159A and T162A ATP-PRT determined at 22  $^{\circ}\text{C}$  and pH 8.5

A., Velocity versus [PRPP] plot for mutant T159A. B., Plot of velocity versus [PRPP] for T162A. The mutant enzymes were added to final concentrations of 600 and 100 nM, respectively for these assays. The PRPP and ATP substrates were at saturating concentrations at 2 mM and 5 mM, respectively. C., Velocity versus [ATP] plot for mutant T159A. D., Velocity versus [ATP] plot for mutant T162A. The mutant enzymes were added to final concentrations of 600 and 100 nM, respectively for these assays. Other details are as noted in the legend to Figure 3.

A. B.

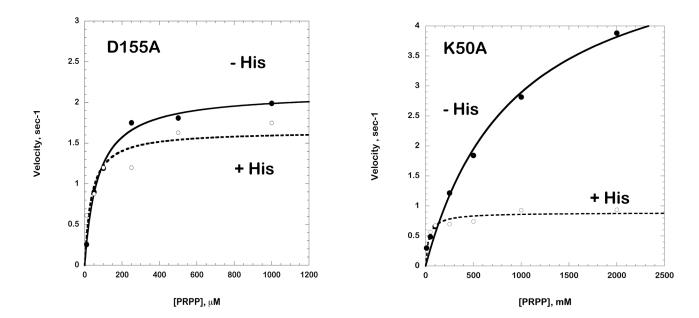


Figure 7. Steady state kinetics of D155A and K50A mutants of L. lactis ATP-PRTase determined at 22  $^{\circ}\text{C}$  and pH 8.5

A., Velocity versus [PRPP] plot for D155A, in the presence (open circles, dotted line) or absence (closed circle, solid line) of 75  $\mu$ M histidine. The mutant enzyme was added to a final concentration of 23.4 nM for these assays. B., Velocity versus [PRPP] for K50A, in the presence (open circles, dotted line) or absence (closed circle, solid line) of 75  $\mu$ M histidine. The mutant enzyme was added to a final concentration of 31.25 nM for these assays. For both enzymes, saturating concentrations of PRPP and ATP substrates were 2 mM and 5 mM, respectively. Other details are as noted in the legend to Figure 3.

# NIH-PA Author Manuscript Table 1 Kinetic Parameters for hexameric and hetero-octameric ATP phosphoribosyl transferases from various species NIH-PA Author Manuscript NIH-PA Author Manuscript

Organism	$\mathbf{k}_{\mathrm{cat}}(\mathbf{s}^{-1})$	K <sub>m</sub> PRPP(μM)	$K_m ATP(mM)$	$K_{i} \operatorname{His}(\mu M)$	K <sub>i</sub> AMP(mM)	Reference
Lactococcus lactis Thermatoga maritima Salmonella typhimurium Salmonella typhimurium Ardpidonsis haliama	2.67±0.27 N.D. <sup>a</sup> N.D. 2.7 N.D.	18,4 ± 3.5 N.D. 67 11 130/570	2.7± 0.26 N.D. 0.2 0.1 0.60/0.51	81.1 350 100 70 45/320 <sup>C</sup>	1.44 N.D. 5 5 N.D.	This work (18) (1) (20,25) (24)

 $^{a}$ N.D., not determined.

 $^{c}$ Reported as an IC50.

barameter values for the two A. thaliana isozymes (ATP-PRT1 and ATP-PRT2) are indicated on either side of the forward slash.

Table 2 Comparison of ATP-PRT kinetic parameters for wild type and mutant hetero-octameric ATP phosphoribosyl transferases from  $Lactococcus\ lactis\$ at  $25^{\circ}$ C, pH  $8.5^{a}$ 

Wild type or mutant substitution	$\mathbf{k}_{\mathrm{cat}}(\mathbf{s}^{-1})$	$K_{m}$ PRPP ( $\mu M$ )	K <sub>m</sub> ATP(mM)	$k_{cat}/K_m^{PRPP}(M^{-1}s^{-1})$
Wild type	2.67±0.27	18.4 ± 3.5	2.7± 0.26	1.5 × 10 <sup>5</sup>
T159A	$1.2 \pm 0.15$	$5151 \pm 1644$	$1.07 \pm 0.23$	$2.3 \times 10^{2}$
T162A	$0.84 \pm 0.08$	$901\pm211$	$1.05 \pm 0.31$	$9.3 \times 10^{2}$
D155A	$2.83 \pm 0.15$	$71.2 \pm 5.2$	$4.7 \pm 0.79$	$3.9 \times 10^{4}$
D155A <sup>b</sup>	$1.75 \pm 0.14$	$37.6 \pm 13.7$	$N.D.^{C}$	$4.7 \times 10^4$
K8A	$1.07\pm0.02$	$90 \pm 6$	$8.45\pm 1$	$1.2 \times 10^{4}$
K50A	$6.16 \pm 0.48$	$940 \pm 193$	$5.0 \pm 0.7$	$6.6 \times 10^{3}$
K50A <sup>b</sup>	$0.95 \pm 0.05$	$36.3\pm 12$	N.D.	$2.6 \times 10^{4}$
S140A <sup>d</sup>	N.D.	N.D.	N.D.	N.D.

 $<sup>^{</sup>a}\mathrm{Each}$  value represents the mean and standard deviation of at least three replicates.

 $<sup>^{</sup>b}$  Parameters determined in the presence of 75  $\mu M$  histidine.

 $<sup>^{</sup>c}$ N.D., not determined.

 $<sup>\</sup>ensuremath{d_{\mathrm{This}}}$  mutant was unstable and its kinetic parameters could not be determined.