Altering the Purine Specifcity of Hypoxanthine-Guanine-Xanthine Phosphoribosyltransferase from *Tritrichomonas foetus* by Structure-Based Point Mutations in the Enzyme Protein[†]

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ABSTRACT: The hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRTase) from *Tritrichomonas foetus* has been proven to be a target for potential anti-tritrichomonial chemotherapy. Using a structure-based approach, the base-binding region of the active site of this enzyme, which confers unique purine base specificity, was characterized using site-directed mutagenesis. Determining the roles of different active-site residues in purine specificity would form the basis for designing specific inhibitors toward the parasitic enzyme. A D163N mutant converts the HGXPRTase into a HGPRTase, which no longer recognizes xanthine as a substrate, whereas specificities toward guanine and hypoxanthine are unaffected. Apparently, the side-chain carboxyl of Asp163 forms a hydrogen bond through a water molecule with the C2-carbonyl of xanthine, which constitutes the critical force enabling the enzyme to recognize xanthine as a substrate. Mutations of Arg155, which orients and stacks the neighboring Tyr156 onto the bound purine base by forming a salt bridge between itself and Glu11, result in drastic increases in the K_ms for GMP and XMP (but not IMP). This change leads to increased k_{cat}s for the forward reactions with guanine and xanthine as substrates without affecting the conversion of hypoxanthine to IMP. Thus, the apparent dislocation of Tyr156, resulted from mutations of Arg155, bring little effect on the hydrophobic interactions between Tyr156 and the purine ring. But the forces involved in recognizing the exocyclic C2-substituents of the purine ring, which involve the Tyr156 hydroxyl, Ile157 backbone carbonyl, and Asp163 side-chain carboxyl, may be weakened by the shifted conformation of the peptide backbone resulted from loss of the Glu11−Arg155 salt bridge. The conserved Lys134 was proven to be the primary determinant in conferring the specificity of the enzyme toward 6-oxopurines. By substituting the lysine residue for a serine, which can potentially hydrogen bond to either an amino or an oxo-group, we have successfully augmented the purine specificity of the enzyme. The K134S mutant recognizes adenine in addition to hypoxanthine, guanine, and xanthine as its substrates. Adenine and hypoxanthine are equivalent substrates for the mutant enzyme with similar K_ms of 34.6 and 38.0 μM, respectively. The catalysis of an adenine phosphoribosyltransferase reaction by this mutant enzyme was further demonstrated by the competitive inhibition of AMP with an estimated K_{is} of 25.4 μM against α-D-5-phosphoribosyl-pyrophosphate (PRPP) in converting hypoxanthine to IMP. We have thus succeeded in using site-directed mutagenesis to convert *T. foetus* HGXPRTase into either a HGPRTase or a genuine AHGXPRTase.

Tritrichomonas foetus, an anaerobic flagellated protozoan, which causes urogenital trichomoniasis in cattle (*1*, *2*), lacks de novo purine nucleotide synthesis and relies primarily on its hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRTase) for salvaging exogenous purine bases to replenish its purine nucleotide pool (*3*). This enzyme has been proven to be a target in *T. foetus* for potential anti-tritrichomonial chemotherapy (*4*). The purine phosphoribosyltransferases (PRTases) form a family of enzymes that catalyzes transfer of the 5-phosphoribosyl moiety from α-D-5-phosphoribosyl-1-pyrophosphate (PRPP)[1] to the imidazole N-9 of a purine base to form the corresponding purine nucleotide. Although there is little sequence homology among these enzymes, their crystal structures reported thus far suggest a common core of five parallel β-strands surrounded by three or four α-helices resembling a typical Rossman dinucleotide binding fold among the type I PRTases (*5*−*9*). These type I enzymes all consist of a highly conserved 13 amino acid PRPP-binding motif near the C-terminus of the third β-strand in the core. Type I purine PRTases from the eukaryotes have varied purine base specificities. Mammalian, yeast (*10*), and *Schistosoma mansoni* (*11*) purine PRTases recognize both hypoxanthine and guanine as substrates, whereas the enzyme from *Giardia lamblia* utilizes only guanine (*12*). *T. foetus* (*13*), *Toxo-*

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[1] Abbreviations: HGXPRTase, hypoxanthine-guanine-xanthine phosphoribosyltransferase; HGPRTase, hypoxanthine-guanine phosphoribosyltransferase; PRPP, α-D-5-phosphoribosyl-1-pyrophosphate; PPi, pyrophosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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plasma gondii (*14*), and *Plasmodium falciparum* (*15*) each has an HGXPRTase that recognizes all three purine bases. The *T. foetus* HGXPRTase has the most unique purine base specificity, because it demonstrates similar catalytic efficiencies (k_{cat}/K_m) of 2.92, 1.03, and 0.80 μM^{-1} s^{-1} for hypoxanthine, guanine, and xanthine, respectively (*13*). This relatively nondiscriminative acceptance of xanthine as a bona fide substrate may constitute an opportunity for selective inhibition of this enzyme in view of the failure of mammalian HGPRTases in recognizing xanthine at all.

The crystal structures of purine PRTases reported thus far contain in each of the active sites a core PRPP-binding domain, which is highly conserved, and a smaller domain involved in binding to the purine moiety in the purine nucleotide (*5*−*9*). This region shows some variations in amino acid residues among different purine PRTases that may constitute the basis for various purine base specificities. Most of the structures include the nucleotide as ligand, and the present understanding of the binding of the purine base is extended from the information on purine nucleotide bindings in these structures.

The purpose of the present investigations is to use site-directed mutagenesis of *T. foetus* HGXPRTase to verify if some of the varied amino acid residues in the purine-binding pocket are indeed involved in conferring such a broad purine specificity to the enzyme, and whether by changing some of the residues, one can alter its specificity. By similar experimental approaches, we intended also to demonstrate if the well-conserved lysine residue among all the type I purine PRTases (Lys134 in *T. foetus* HGXPRTase and Lys165 in human HGPRTase) provides the main restriction on purines with exocyclic O6 as the only substrates and thus excluding adenine from being a substrate for these enzymes.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals used in the present studies, including hypoxanthine, guanine, xanthine, adenine, IMP, GMP, XMP, AMP, PPi, and the tetrasodium salt of PRPP, were purchased from Sigma Chemical Co. (St. Louis, MO) and are of the highest purities available. Xanthine oxidase (1 unit/mg protein) was from Boehringer Mannheim (Indianapolis, IN), and guanase (0.1 unit/mg protein) was from Sigma Chemical Co.

Site-Directed Mutagenesis of the Cloned Gene Encoding T. foetus HGXPRTase. Oligonucleotide primers were designed and synthesized to generate various site-directed mutants of the encoding gene for *T. foetus* HGXPRTase. The plasmid containing the full-length gene encoding *T. foetus* HGXPRTase (pBTfprt) was isolated and purified from the transformed *Escherichia coli* strain pBTfprt/DH5α (*16*) and used as template for site-directed mutagenesis, using the kit from Stratagene. Following the polymerase chain reaction (PCR) with the synthesized primer, each mutant DNA plasmid was transformed into an *E. coli* mutant strain Sϕ606 (*16*), amplified, isolated, and sequenced for verification of the site-directed mutation.

Expression and Purification of the Recombinant Enzymes. The pBTfprt expression plasmid transformed *E. coli* Sϕ606 cells were cultured, and the expression of *T. foetus* HGXPRTase gene in the plasmid was induced in the low-phosphate culture medium as previously described (*16*). The recombinant *T. foetus* HGXPRTase wild-type and the site-directed mutants were each purified to homogeneity from the transformed cells as described by Beck and Wang (*17*) with some minor modifications (*18*). The purified samples were each examined for purity in sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and stored at −70° without detectable activity loss during the first 4 months.

Steady-State Kinetic Study of the Enzyme-Catalyzed Reactions. The nucleotide synthesis from PRPP and hypoxanthine, guanine, and xanthine were each monitored spectrophotometrically at 245, 257.5, and 247 nm, respectively, using a Beckman DU-7 spectrophotometer equipped with a kinetics accessory as previously described (*13*). The assays were conducted in 100 mM Tris-HCl, pH 7.0, and 12 mM $MgCl_2$ at 37 °C. The extinction coefficients for formation of the nucleotides from hypoxanthine, guanine, and xanthine under these conditions were 1770, 6000, and 5660 M^{-1}, respectively. The pyrophosphorolysis of IMP, GMP, or XMP was indirectly monitored by measuring uric acid formation spectrophotometrically at 293 nm, with an extinction coefficient of 12 000 M^{-1}, as described earlier (*13*). Each K_m and V_{max} value was determined from multiple fixed substrate concentrations versus the varying concentrations of the other substrate as indicated previously (*13*). The assay for adenine phosphoribosyltransferase activity was also by spectrophotometric measurements. The formation of AMP from adenine was assayed at 256 nm, at which the change in extinction coefficient from adenine to AMP is 2900 M^{-1} cm^{-1}. For each of the mutant enzymes, the K_m and V_{max} values for PRPP and PPi were also determined versus hypoxanthine and IMP, respectively.

Analysis of the Kinetic Data. Data on the initial rates of the enzyme-catalyzed reaction were fitted into the equations for equilibrium ordered (*19*), using the kinetics software from BioMetallics, Inc. (k_{cat}), and IntelliKinetics (KinetAsyst) with Gauss−Newton analysis (*13*). The Lineweaver−Burk plots and the kinetic constants were obtained using the weighted linear regression.

Reaction with Phenylglyoxal. The enzyme protein (2 μM) in 50 mM Tris-HCl buffer, pH 7.4, 6 mM $MgCl_2$ and 1 mM dithiothrietol, was incubated with 5 mM phenylglyoxal at 25 °C. At different time intervals, aliquots were assayed for HGXPRTase activity. Parallel experiments were conducted with 50 mM GMP in the reaction mixture to block the reaction with phenylglyoxal (*13*).

RESULTS AND DISCUSSION

Comparing the Purine-Binding Sites between T. foetus HGXPRTase and Human HGPRTase. The crystal structure of *T. foetus* HGXPRTase demonstrates the presence of a number of hydrogen bonds between several amino acid residues in lobe 2 of the protein and the guanine moiety of the bound GMP (*7*). The NZ of Lys134 and the backbone amide of Ile157 both form hydrogen bonds with O6 of the purine ring, presumably for distinguishing those purines with exocyclic O6 (hypoxanthine, guanine, and xanthine) from that with exocyclic N6 (adenine) (Figure 1A). The purine N1 and guanine exocyclic N2 are within hydrogen-bonding distances from the backbone carbonyl of Ile157. The guanine exocyclic N2 can also form a hydrogen bond with

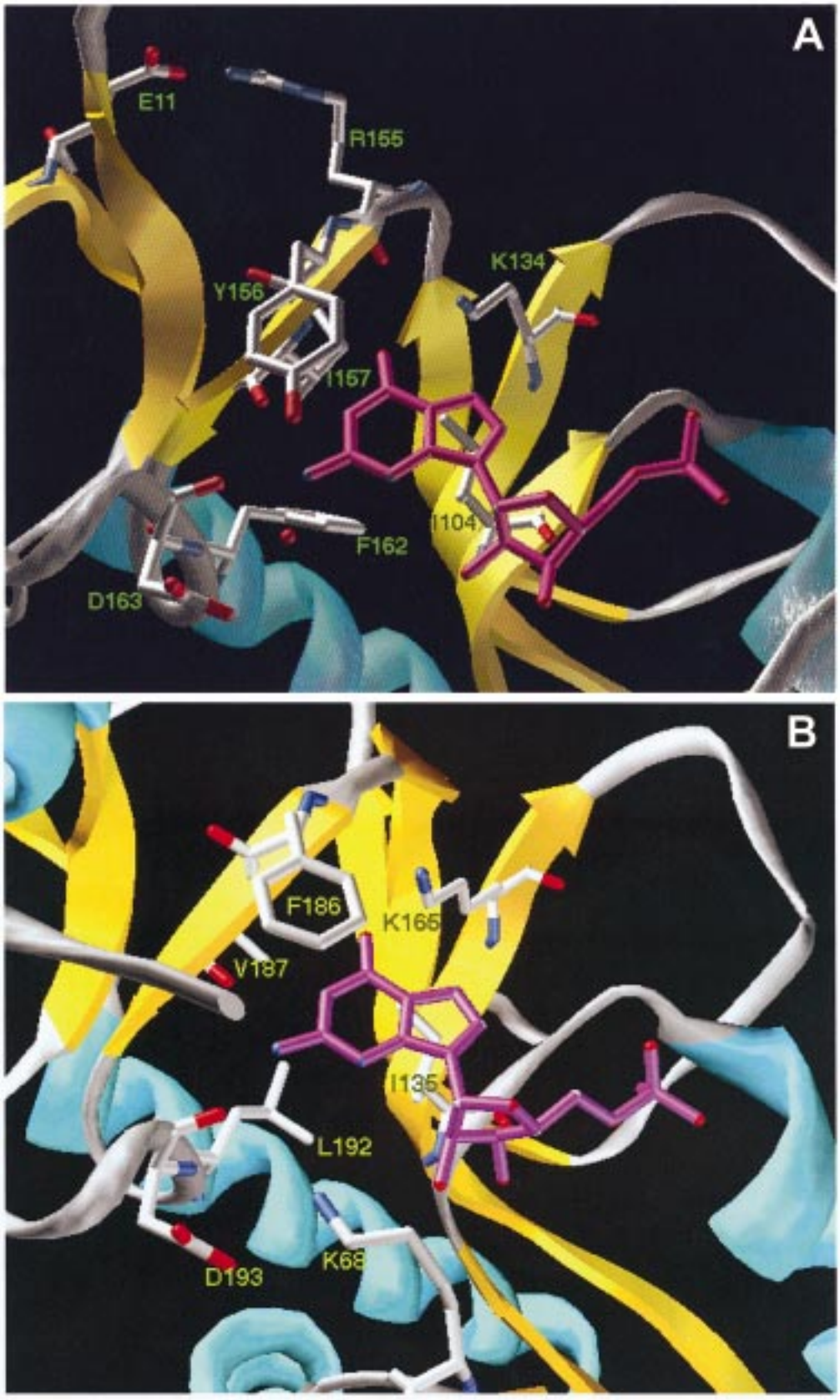

FIGURE 1: Comparison of the active-site geometries of GMP-bound forms of *T. foetus* HGXPRTase (*7*) (A) and human HGPRTase (*8*) (B).

the main-chain oxygen of Asp163, whereas the carboxylic O of Asp163 forms a link with the xanthine exocyclic O2 by hydrogen bonding through a water molecule. Tyr156, stacked on top of the purine ring by apparent hydrophobic interactions, might form a hydrogen bond via its hydroxyl group with either guanine N2 or xanthine O2. But these interactions could be quite limited, because it has been observed that the hydroxyl group of tyrosine shows significant preference for interactions within the plane of its own phenolic ring (*20*). On the other hand, the hydroxyl group

of Tyr156 could potentially form a hydrogen bond with the main-chain carbonyl of Asp163, latching the aromatic group in place stacked above the base. Hence, the primary interaction of Tyr156 with the purine ring would be through p−p binding forces. The hydrophobic forces from the side chains of Ile104 and Phe162 on the opposite side further stabilize binding of the purine ring.

In the GMP-bound active site of human HGPRTase (*5*), the exocyclic guanine O6 hydrogen bonds with the backbone amide of Val187 and the NZ of Lys165, similar to that observed in *T.foetus* HGXPRTase (Figure 1B). The exocyclic N2 of guanine forms hydrogen bonds with the main-chain carbonyl oxygens of Val187 and Asp193, whereas the relatively high-binding affinity of hypoxanthine was attributed to the occupancy of a water molecule in the C2 pocket of the purine base (*5*). The carboxylic O of Asp193 is, however, too far removed from this C2 pocket (∼6 Å) for a potential bond formation with the exocyclic O2 of xanthine through a water molecule, which may explain why xanthine is not a substrate for the human enzyme (Figure 1B). A primary contribution toward the nonacceptance of xanthine could be the presence of Lys68 near that pocket. The NZ of Lys68 is close to the N3 group of GMP in the human HGPRTase structure (*5*), and since this is protonated in the case of xanthine, there would be no favorable H-bond or electrostatic interactions between xanthine (or XMP) and the lysine. A phenylalanine residue 186 is stacked on top of the purine ring. Below the purine ring, there are Ile135 and Leu192 that provide the apparent hydrophobic force for stabilizing the purine binding. However, a recent observation that a L192T mutant of the human enzyme had lower apparent K_m values for hypoxanthine and guanine without appreciable change in catalytic efficiencies raised some doubt on the relative contribution from the hydrophobic interactions between Leu192 and the purine in stabilizing the latter (*9*).

These comparisons between purine bindings to the active sites in human HGPRTase and *T. foetus* HGXPRTase revealed differences between the two enzymes. The parasite enzyme not only lacks the amino group of Lys68 in close proximity to N3 of the base as in the human HGPRTase (*5*), but also the corresponding Thr47 is placed on a loop that is positioned away from the active site. This leaves the entire edge of the base exposed to solvent in the *T. foetus* HGXPRTase structure. We hypothesize two mechanisms that may enable xanthine to become only a substrate for the *T. foetus* enzyme. Either the hydrogen bonding of the carboxylic oxygen of Asp163 with the exocyclic O2 or the protonated N3 of xanthine through a water molecule or the hydrogen bond between the hydroxyl of Tyr156 and xanthine O2 may have made a major contribution to this expanded substrate specificity.

Site-Directed Mutants of T. foetus HGXPRTase. There were six categories of site-directed mutants of *T. foetus* HGXPRTase prepared in the current investigations. They were mutants with individual amino acid replacements at Asp163, Arg155, Tyr156, Ile104, Phe162, and Lys134, respectively. For each category of mutants, there are (a) D163N and D163E; (b) R155K and R155E; (c) Y156F and Y156W; (d) I104G; (e) F162L; and (f) K134A, K134S, and K134Q. Each of the recombinant mutant enzymes was purified and analyzed in SDS−PAGE. The results presented in Figure 2 demonstrate that all the mutant enzyme proteins were purified to near homogeneity prior to the kinetic analysis of the enzyme-catalyzed reactions.

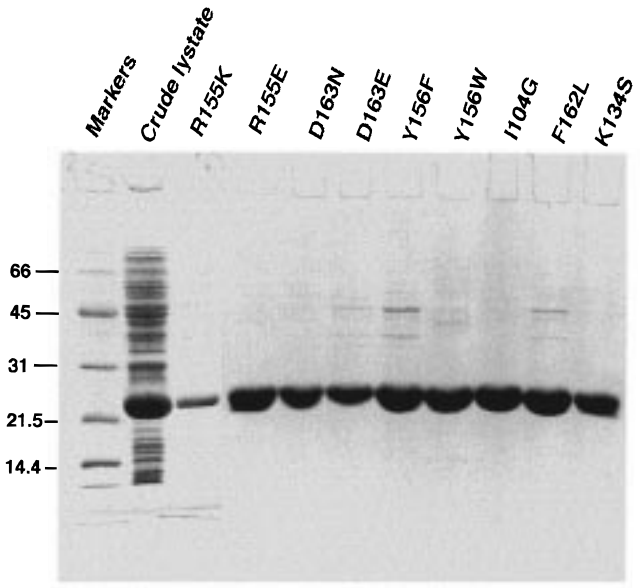

FIGURE 2: SDS−PAGE analysis of the purified *T. foetus* HGXPRTase mutant proteins.

Mutant D163N Is an HGPRTase. The K_m values for hypoxanthine, guanine, and xanthine in the *T. foetus* HGXPRTase-catalyzed forward reaction are 3.0 ± 0.5, 2.4 ± 0.7, and 6.1 ± 0.8 μM, respectively. In the reversed reaction, the K_m values for IMP, GMP, and XMP are 12.1 ± 0.7, 23.3 ± 4.7, and 31.5 ± 7.1 μM, respectively (*13*). As the three purine substrates differ in the substituent at C2 position but show no major differences in the K_m values, a specific residue involved in specific binding to the C2 position of purine may be missing from the active site, or the binding energy arising from the interaction of the residue(s) in the active site with the C2 position in purine could be very low. In *T. foetus* HGXPRTase, Asp163 interacts with the exocyclic N2 of GMP through its main-chain carbonyl as well as the side-chain carboxyl through a water molecule. This residue is highly conserved among all of the purine PRTases and would seem unlikely to influence the purine specificities of these enzymes. However, a mutation of this residue to Asn in *T. foetus* HGXPRTase resulted in a mutant enzyme (D163N) which no longer recognizes xanthine as a substrate even when tested at a concentration of 300 μM (Table 1), whereas it still acts on guanine and hypoxanthine, with only slightly augmented K_m values. A similar effect is also shown on the purine nucleotide substrates in the reversed reaction, wherein the K_m of XMP in the D163N mutant-catalyzed reaction is increased 9-fold, and the K_ms for IMP and GMP are only about 3-fold higher than that in the wild-type enzyme-catalyzed reaction (Table 2). These results suggest that the side-chain carboxyl of Asp163 plays a critical role in recognizing the C2-carbonyl of xanthine and XMP by forming a hydrogen bond through a water molecule (*7*). Thus, the loss of xanthine recognition by the mutant enzyme could be due to the anticipated failure of the carboxamide group of Asn163 to link up with the C2-carbonyl of xanthine through a water molecule. Unlike guanine, xanthine has an additional proton at the N3 position, which may direct the water-mediated Asp163 carboxyl to interact with both the C2 carbonyl as well as the N3 proton in xanthine. The loss

Table 1: Comparison of the Kinetic Constants of the Forward Reactions for Various Site-Directed Mutants and Wild-Type HGXPRTase

	hypoxanthine		guanine		xanthine	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
W/T	3.0 ± 0.5	8.9 ± 0.5	2.4 ± 0.7	2.5 ± 0.2	6.1 ± 0.8	4.8 ± 0.8
D163N	4.9 ± 0.8	8.9 ± 0.7	9.1 ± 1.1	2.2 ± 0.1	>300	-
D163E	3.0 ± 0.4	8.5 ± 1.4	5.0 ± 0.8	2.4 ±0.1	15.8 ±1.0	5.4 ± 1.3
R155K	3.6 ± 0.8	11.0 ± 0.5	2.9 ± 0.5	13.0 ± 0.6	6.0 ± 0.7	19.0 ± 3.8
R155E	2.1 ± 0.5	10.2 ± 0.8	3.0 ± 0.2	26.0 ± 1.9	95.8 ± 18.4	33.0 ± 2.6
Y156W	1.6 ± 0.3	7.2 ± 0.9	1.6 ± 0.2	1.4 ± 0.1	8.8 ± 1.2	4.6 ± 0.3
Y156F	1.8 ± 0.3	9.7 ± 0.8	1.9 ± 0.3	6.6 ± 0.5	9.1 ± 1.3	10.6 ± 0.9
I104G	12.4 ± 2.0	6.7 ± 0.7	15.4 ± 3.2	1.6 ± 0.1	83.6 ± 9.3	3.3 ± 0.2
F162L	1.8 ± 0.2	1.2 ± 0.1	1.9 ± 0.1	0.7 ± 0.1	7.4 ± 0.9	1.1 ± 0.1

Table 2: Comparison of the Kinetic Constants of the Reverse Reactions for Various Site-Directed Mutants and Wild-Type HGXPRTase

	IMP		GMP		XMP	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
W/T	12.1 ± 0.7	0.20 ± 0.03	23.3 ± 4.7	0.9 ± 0.1	31.5 ± 7.1	0.17 ± 0.02
D163N	37.1 ± 14.3	0.14 ± 0.03	79.2 ± 25.3	0.66 ± 0.08	262.9 ± 63.3	0.11 ± 0.02
D163E	ND[a]	ND	ND	ND	29.4 ± 6.1	0.14 ± 0.01
R155K	11.2 ± 2.2	0.18 ± 0.03	181.8 ± 6.5	0.82 ± 0.1	648.2 ± 129.0	0.16 ± 0.01
R155E	9.5 ± 1.1	0.08 ± 0.01	289.8 ± 43.6	0.65 ± 0.08	699.2 ± 113.0	0.25 ± 0.04
Y156W	10.7 ± 2.2	0.13 ± 0.01	29.7 ± 4.5	0.86 ± 0.06	27.8 ± 5.2	0.13 ± 0.02
Y156F	16.5 ± 2.2	0.09 ± 0.01	138.3 ± 19.7	0.76 ± 0.10	118.1 ± 21.9	0.18 ± 0.02
I104G	34.6 ± 5.5	0.08 ± 0.01	75.6 ± 11.2	0.68 ± 0.04	55.4 ± 4.6	0.19 ± 0.02
F162L	ND	ND	19.4 ± 3.2	0.69 ± 0.03	14.9 ± 2.9	0.07 ± 0.01

[a] ND, not determined.

of Asp163 carboxyl can thus convert the HGXPRTase essentially to a HGPRTase. This conclusion is supported by our additional observations on another mutant enzyme D163E. The residue change in this mutant shows no significant effect on the K_ms for xanthine in the forward reaction or for XMP in the reversed catalysis, suggesting that the carboxyl group is indeed essential in position 163 for recognizing xanthine. The D163E mutant also shows no significant change in the K_ms for hypoxanthine, guanine, IMP, and GMP. There is also no appreciable alteration in the kinetic constants for PRPP and PPi in either the D163N or the D163E-catalyzed reactions.

Mutants R155K and R155E Show Reduced Affinity to GMP and XMP and Catalyze the Forward Reactions with Guanine and Xanthine at Accelerated Rates. In a previous investigation, we observed that alkylation of the guanidino group of Arg155 in *T. foetus* HGXPRTase by the specific arginine-modifying agent phenylglyoxal resulted in a total and irreversible inactivation of the enzyme activity (*21*). GMP can block this Arg155 alkylation and enzyme inactivation, though Arg155 is located outside the active site and held together with Glu11 through salt bridges in the lid region of the protein (Figure 1A). However, since the neighboring residue Tyr156 stacks on top of the purine base, a fixed position of Arg155 could be essential for a properly oriented and positioned Tyr156 for a facilitated binding with the purine base. We examined this hypothesis by generating mutants of the HGXPRTase with Arg155 replaced by lysine and glutamic acid. As anticipated, the R155K mutant is no longer inhibited by the phenylglyoxal treatment as shown in Figure 3 (*18*). The K_m and k_{cat} for each of the enzyme substrates, both in the forward and reversed reactions, were determined in the R155K mutant-catalyzed reactions as described in the Materials and Methods. As shown in Table 1, the K_m values for the three nucleobases do not vary from the wild-type enzyme-catalyzed reactions, whereas the k_{cats}

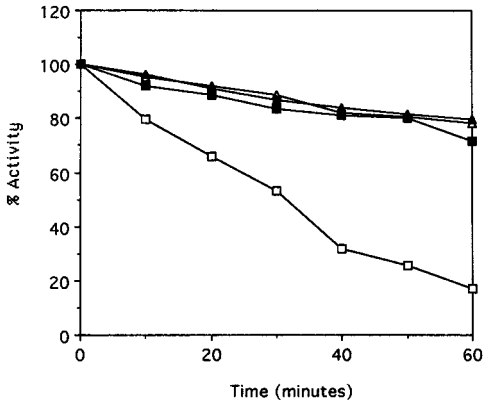

FIGURE 3: Inhibition of *T. foetus* HGXPRTase activity by phenylglyoxal. The procedure of phenylglyoxal treatment and the enzyme activity assay was described in the Materials and Methods. (□) Wild-type HGXPRTase; (△) wild-type HGXPRTase with 50 mM GMP; (■) the R155K mutant; (▲) the R155K mutant with 50 mM GMP.

with guanine and xanthine show a 4−5-fold increase. In the reversed reactions, the K_m for IMP does not change, whereas the K_ms for GMP and XMP are significantly higher than the corresponding wild-type Michaelis constants (Table 2). The k_{cat}s in the reversed direction of the mutant enzyme-catalyzed reaction show no change with any of the three nucleotide substrates. The K_m for PRPP determined in the presence of hypoxanthine turned out to be 45.8 ± 7.5 μM as compared to 39.9 ± 5.6 μM for the wild-type enzyme-catalyzed reaction. The K_m for PPi with IMP as cosubstrate was found to be 236.2 ± 56.6 μM, whereas for the wild-type enzyme, it is 282.9 ± 77.8 μM. Therefore, the bindings of PRPP and PPi are unaffected by the R155K mutation.

In the R155E mutant enzyme-catalyzed reactions, the K_ms for hypoxanthine and guanine show no change, whereas that for xanthine has a 15-fold increase (Table 1). There is no significant change in the k_{cat} for hypoxanthine but there is a

10-fold and a 7-fold increase in the k_{cat} values for guanine and xanthine, respectively. The K_m for IMP has no appreciable change, whereas the K_ms for GMP and XMP are increased by 12- and 22-fold, respectively (Table 2). The k_{cat} values for the three purine nucleotides remain, however, relatively unchanged. The Michaelis constants for PRPP and PPi in the mutant enzyme are again the same as in the wild-type enzyme-catalyzed reactions.

These results from the R155K- and R155E-catalyzed reactions can be summarized as follows; (a) neither mutation exerts any effect on the kinetics of interconversion between hypoxanthine and IMP; (b) neither mutation affects the kinetic constants for PRPP or PPi; (c) the bindings of guanine and xanthine are unaffected by the R155K mutation, but the xanthine binding to R155E is significantly weakened; (d) the conversion of guanine and xanthine to the corresponding nucleotides are greatly accelerated by both mutants; (e) GMP and XMP show much weakened bindings to both mutants. To further determine the affinity of the mutants toward GMP, we conducted product inhibition studies. The K_{is} for GMP was determined against PRPP, at constant saturating concentration of guanine (100 μM). The K_{is}s for GMP in the R155K- and the R155E-catalyzed reactions were determined to be 87.4 $\pm$ 17.3 and 147.9 $\pm$ 21.3 respectively, whereas the corresponding value for the wild-type enzyme is 14 $\pm$ 1.9 (*13*). Thus, the 6-fold increase for the R155K and the 10-fold increase for the R155E mutant in the K_{is} for GMP indicate a loss of affinity for GMP as a result of substituting the critical Arg155 with lysine or glutamic acid. Since the release of nucleotide in the purine PRTase-catalyzed forward reaction has been found to be the rate-limiting step (*19*), it is relatively easy to understand how the weakened bindings of GMP and XMP to the mutant enzymes could lead to greatly accelerated conversion of guanine and xanthine to the corresponding nucleotides. But, it is more difficult to see how the alteration of Arg155, a residue outside of the active site (see Figure 1A), can selectively influence the binding of certain nucleobase or nucleotide to the active site. The crystal structure of *T. foetus* HGXPRTase shows that Arg155 forms a salt-bridge with Glu11 (Figure 1A). When the salt-bridge is partially disrupted in the R155K mutant or more drastically broken in the R155E mutant, there could be a displacement of the peptide backbone containing the neighboring Tyr156, Ile157, and Asp163 residues known to interact with the C2-substituent of the purine substrate. Thus, there could be a displacement of the Tyr156 hydroxyl, the Ile157 backbone carbonyl, and the Asp163 carboxyl as the result of an Arg155 mutation. This could lead to decreased binding of GMP and XMP but not IMP, because the latter does not have a substituent at the C2 position. The bindings of guanine and xanthine are, however, unaffected in the R155K mutant, and only the K_m of xanthine is increased by 15-fold in R155E, a mutant with presumably more drastic changes in the peptide backbone. These results suggest that nucleobase binding may differ from nucleotide binding. In the ordered Bi−Bi mechanism of the HGXPRTase-catalyzed reactions (*13*), nucleobases can bind only to the enzyme−PRPP complex, whereas the nucleotides bind to the free enzyme. The PRPP binding may induce structural modifications in the enzyme (*19*) and help bring the residues involved in base bindings into appropriate orientations. The relatively low K_m values for the purine bases when compared with those of the nucleotides suggest a closed conformation of the active site for binding the purine base and an open conformation for binding to the nucleotide as seen in the crystal structure of the enzyme complexed with the nucleotide (*7*).

In the R155E mutant, a 15-fold decreased xanthine binding could mean a significant displacement of Asp163, which is specifically involved with xanthine binding, even in the PRPP−enzyme complex. The flexibility in the purine-binding peptide backbone would also dictate to a large measure the specificity of the enzyme. Such flexibility would translate to minor structural alignments in order to accommodate either an amino or a carbonyl group at the C2 position in the purine moiety. Further upstream to this peptide backbone, the HGXPRTase has a Glu at position 152, whereas in the human HGPRTase and some other purine PRTases, there is a conserved proline residue instead. A proline at this position would expectedly lower the flexibility of the downstream purine-binding peptide and make these enzymes less accommodating to different purine base structures.

Mutant Y156F Has a Weakened Binding of GMP and XMP. To determine the role of Tyr156 in the binding of purine bases to HGXPRTase, we created two mutants, Y156F and Y156W, because human HGPRTase has a Phe (*5*) and *Toxoplasma gondii* HGXPRTase has a Trp residue (*6*) at the corresponding location. In the case of the Y156W mutant, there is no significant change in the kinetic constants for all the enzyme substrates both in the forward and the reversed directions as compared to the wild-type enzyme-catalyzed reactions (Tables 1 and 2). Thus, the indole ring of Trp156 appears to be capable of interacting with the substrates equally well as the Tyr of the wild-type. The Y156F mutant (Table 1) shows a 2−3-fold increase in the k_{cat} values with guanine and xanthine and a 2−3-fold increase in the K_ms for GMP and XMP in the reverse reaction (Table 2). In comparison, a mutant of the neighboring Arg155 (R155E) showed much more drastic changes in the K_ms for GMP and XMP. This contrast indicates that the role of the hydroxyl of Tyr156 in hydrogen bonding to the C2 substituent in purine is quite minimal. This is somewhat expected since the interaction of the hydroxyl with the purine moiety is not in the same plane (see Figure 1A).

Isoleucine 104 Stabilizes Purine Bindings in General. As seen in the crystal structure of HGXPRTase, some of the hydrophobic stacking interactions with GMP arise from the side-chain groups from Ile104 and Phe162 from below (see Figure 1A). Mutant I104G, as shown in Table 1, shows increased K_m values for all three purine bases. Hypoxanthine shows a moderate 4-fold increase, guanine has a 6-fold increase in its K_m, and the K_m for xanthine is 13-fold higher than that for the wild-type enzyme. The K_ms for the nucleotides showed only a 2−3-fold increase in general. Thus, the Ile104 residue in *T. foetus* HGXPRTase appears to be a stabilizing factor for bindings of purine bases without discriminating the detailed purine base structures. The role of Phe162 in *T. foetus* HGXPRTase was scrutinized by creating a mutant with Leu162 equivalent to the Leu192 residue in human HGPRTase. The F162L mutant had no significant effect on the K_m of either the bases or the nucleotides, suggesting that the presence of a Phe instead of a Leu residue at position 162 of the *T. foetus* enzyme has

Table 3: Kinetic Constants of the Mutants of Lys134

	W/T		K134A		K134S		K134Q	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
hypoxanthine	3.0 ± 0.5	8.9 ± 0.5	52.7 ± 8.5	15.7 ± 2.9	38.0 ± 4.3	14.0 ± 1.6	1.4 ± 0.2	9.5 ± 0.7
guanine	2.4 ± 0.7	2.5 ± 0.2	154.6 ± 23.0	10.7 ± 2.1	ND	ND	ND	ND
xanthine	6.1 ± 0.8	4.8 ± 0.8	191 ± 39.4	4.1 ± 0.3	123.4 ± 23.0	7.4 ± 0.5	ND	ND
adenine	>200		53.9 ± 1.9	0.028 ± 0.003	34.6 ± 2.0	0.096 ± 0.005	198.6 ± 32.9	0.025 ± 0.002
IMP	12.1 ± 0.7	0.19 ± 0.03	185.6 ± 11.3	0.22 ± 0.01	40.3 ± 2.9	0.13 ± 0.02	13.1 ± 2.5	0.21 ± 0.03

[a] ND, not determined.

no bearings to the expanded purine base specificity.

From the above results, it is clear that factors dictating the purine base specificity in *T. foetus* HGXPRTase are quite complex. Highly intricate local environment around the C2-position of the base determines the characteristic specificity of the individual PRTases. The capability of minor structural alignments and recruitment of bridging water molecules to accommodate purine substrates with different substituents at the C2 position can be amplified to a significant extent in the specific recognition of a new purine substrate such as xanthine. The ability of the parasite enzyme to accomplish fine structural adjustments can be exploited in designing specific inhibitors, especially since this enzyme has a relatively open structure near the edge of the base-binding region, unlike that in the human HGPRTase.

K134S Mutant Is a Genuine Adenine Phosphoribosyltransferase (APRTase). In all the reported purine PRTase structures (*5*−*9*), a conserved lysine is observed to be binding to the O6 of the purine ring through a hydrogen bond and has been postulated to be influential in allowing only 6-oxo purines and not 6-amino purine such as adenine to bind to the active site. To evaluate whether Lys134 in *T. foetus* HGXPRTase plays the specific role in determining the enzyme specificity toward only the purine substrates with exocyclic O6, we designed mutants at this specific position. The kinetic constants listed in Table 3 indicate that when Lys134 is substituted by alanine, the K_ms for all three purine bases show a sharp 17−64-fold increase over the wild-type enzyme. There is also a 15-fold increase in the K_m for IMP. The most dramatic observation was of an adenine phosphoribosyltransferase (APRTase) reaction catalyzed by the K134A mutant enzyme. The apparent K_m for adenine, determined at 2 mM PRPP, was 54.0 ± 1.9 μM with a k_{cat} of 0.028 ± 0.003 s^{-1}. The wild-type enzyme fails to act on adenine even at a concentration of adenine at 200 μM with 20 μg of the pure enzyme protein in the assay mixture. When the Lys134 residue was changed to a serine, the K_m for adenine was lowered to 34.6 ± 2.0 μM, with an enhanced k_{cat} of 0.096 ± 0.005 s^{-1} (Figure 4). The K_ms for hypoxanthine and xanthine were 12−20-fold higher than those for the wild-type enzyme catalyzed reactions (Table 3). The K_m for IMP in the K134S mutant enzyme-catalyzed reverse reaction was only around 3-fold higher than that for the wild-type enzyme. Thus, concurrent to the 5-fold increase in the catalytic efficiency of the K134S mutant enzyme for the APRTase reaction over the K134A mutant enzyme, there is also the coordinated increase in binding of the K134S mutant to hypoxanthine, xanthine, and IMP. This would be expected since the void in the active site created by alanine is replaced by a serine, which could act both as a hydrogen bond acceptor with 6-oxopurine substrates as well as a donor with

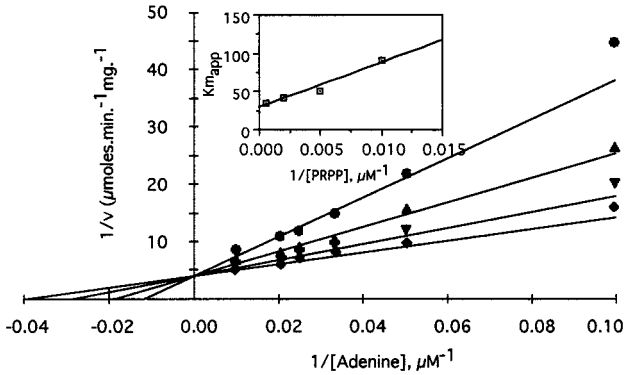

FIGURE 4: Initial velocity patterns for the APRTase reaction catalyzed by the K134S mutant, with adenine as the variable substrate (10−100 μM) at different fixed concentrations of PRPP (0.1−2 mM). All other conditions were as described in the Materials and Methods. (Inset) Replot of K_{mapp} against reciprocal micromolar concentrations of PRPP.

the 6-aminopurine substrate. The interaction between the serine hydroxyl and the 6-amino group of adenine is most likely facilitated by an intermediate water molecule, in order that other enzyme−substrate interactions are conserved as with the 6-oxo purines. Surprisingly, K134Q mutant recognizes adenine only poorly, with a high K_m of 198.6 ± 32.9 μM and a k_{cat} of 0.025 ± 0.002 s^{-1}. On the other hand, the K_ms for hypoxanthine and IMP are similar to the wild-type values (Table 3). It is possible that the stereochemical orientation of the carboxamide group in Gln134 prevents optimal hydrogen bonding to the N6 of adenine, while still maintaining the orientation of the NZ-O6 hydrogen bond with the 6-oxopurines. However, unlike the wild-type enzyme, the K134Q mutant enzyme is still capable of converting adenine to AMP at a detectable rate, suggesting that the interaction of the Gln134 carboxamide group with N6 of adenine may be lower than Ser134, but is not totally absent.

In a separate study, we used product inhibition experiments to determine affinities of the Lys134 mutants toward AMP. The K_is for AMP was determined against varying concentrations of PRPP in the presence of an excessively high concentration of hypoxanthine (200 μM). As shown in Figure 5, the data for K134S mutant enzyme fit well with the equation for competitive inhibition of AMP against PRPP with a calculated K_is value of 25.4 ± 3.6 μM. The K_is for AMP in the K134A and K134Q catalyzed forward reactions were determined under the same experimental conditions and were calculated to be 348.8 ± 17.4 and 247.2 ± 22.6 μM, respectively. AMP at concentrations up to 200 μM demonstrated no effect on the wild-type enzyme catalyzed forward reactions. We have, thus, successfully converted a type I purine phosphoribosyltransferase, which recognizes only 6-oxopurines as substrates, into an AHGXPRTase by

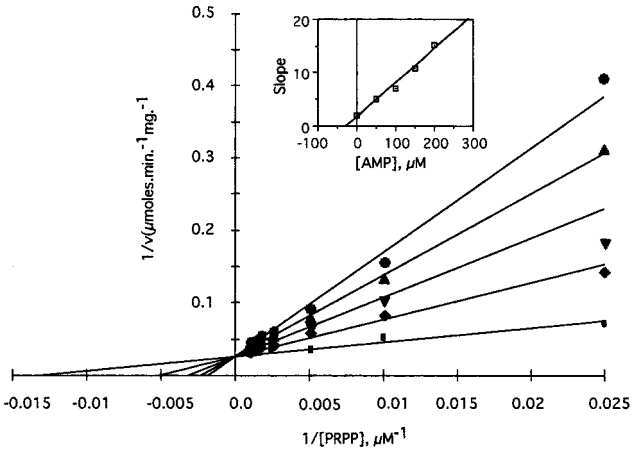

FIGURE 5: AMP inhibition of the forward reaction catalyzed by the K134S mutant, with PRPP as the variable substrate. The concentration of hypoxanthine was kept constant at 200 μM, and all other conditions were as described in Methods. (Inset) Replot of slope against micromolar concentrations of AMP to determine its K_{is} value.

a single amino acid change in the enzyme protein. This result has confirmed the previous postulation that Lys134 in *T. foetus* HGXPRTase performs indeed the function of confining only the 6-oxopurines as its substrates, and serves a primary deterrent toward binding of adenine.

Thus, by using a structure-based approach, we have successfully broadened the purine specificity of the *T. foetus* enzyme from HGXPRTase to APRTase in the K134S mutant and restricted the purine specificity by eliminating the XPRTase activity in the D163N mutant. As far as we are aware, both of these changes in purine specificity of the type I purine phosphoribosyltransferase through single-point mutations are unprecedented and may provide a starting point for further in-depth investigations on the structure and function of this family of enzymes.

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