Converting the Guanine Phosphoribosyltransferase from *Giardia lamblia* to a Hypoxanthine-guanine Phosphoribosyltransferase*

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Received for publication, August 10, 2000, and in revised form, August 30, 2000
Published, JBC Papers in Press, September 6, 2000, DOI 10.1074/jbc.M007239200

Purine phosphoribosyltransferases (PRTases)* are crucial purine salvage enzymes for many species of parasitic protozoa (1, 2), which in general lack the ability to synthesize purine nucleotides *de novo*. Purine PRTases catalyze the Mg $^{2+}$-dependent synthesis of purine nucleotides by conjugating purine bases such as adenine, hypoxanthine, guanine, and xanthine with the phosphoribosyl group of α-d-5-phosphoribosyl-1-pyrophosphate (PRPP) by an ordered Bi-Bi mechanism (3–6). Crystall structures of the type I PRTases share a common Rossmann’s fold and a hood composed of mostly anti-parallel β sheets, between which lies the active site of the enzyme (7–13).

*Giardia lamblia* is an anaerobic binucleate flagellated protozoan causing intestinal infection in mammals (14). Two separate parallel pathways providing the major means of synthesizing AMP and GMP are catalyzed by adenine phosphoribosyltransferase and guanine phosphoribosyltransferase (GPRTase), respectively, constituting the primary routes of purine salvage in this organism (15). The lack of interconversion between AMP and GMP has rendered either of the two purine salvage enzymes potential targets for anti-giardiasis chemotherapy (1, 15).

G. lamblia GPRTase shows little sequence homology with human hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) and other known purine PRTases (16). It converts only guanine to its corresponding nucleotide with an unusually high $K_m$ of 16.4 ± 1.8 μM and a very high $k_{cat}$ of 76.7 ± 2.5 s$^{-1}$ compared with those of other known purine PRTase-catalyzed reactions (5). It exhibits a very low catalytic activity with hypoxanthine as substrate ($K_m >200$ μM) and has no apparent binding with xanthine at all. It is the only specific GPRTase, as far as we are aware, that has been thoroughly identified thus far.

The crystal structure of *G. lamblia* GPRTase has recently been solved at a 1.75-Å resolution (12). The enzyme protein, complexed with a transition state GMP analog, immucillin G phosphate together with Mg$^{2+}$ and pyrophosphate, showed the presence of a common Rossmann’s fold and the typical hood domain present among all type I PRTases (7–13). Although primary interactions between the purine substrate and the amino acid residues at the active site are highly conserved among all the purine PRTases with known crystal structures (7, 11, 17), minor structural differences in the hood region have been noted between the GPRTase and human HGPRTase (7). In *Giardia* GPRTase, the backbone carbonyls of Asp-181 and Asp-187 interact with the exocyclic N2 of guanine, with estimated distances of 2.7 and 3.1 Å, respectively (12). The purine ring is stacked between two aromatic residues, Trp-180 and Tyr-127. Again, the hydrophobic ring stacked on top of the purine ring has been well conserved among all the PRTases. A similar Trp residue is present in *Toxoplasma gondii* HGXPRTase (10), whereas a Tyr residue is present at the corresponding position in *Trichomonas foetus* HGXPRTase (13) and a Phe residue in human HGPRTase (7). The presence of Tyr-127 in the GPRTase is, however, highly unusual, because a well conserved Ile or Leu residue has been identified at the corresponding position among all the other PRTases (7). It appears from the three-dimensional structure of GPRTase (see Fig. 1A) that Tyr-127 could cause extra steric hindrance to purine binding compared with the corresponding Ile-135 in human HGPRTase (Fig. 1B).

Another well conserved residue in the purine-binding pocket...
among type I purine PRTases is the Leu-192 in human HG-
PRTase showing a close nonpolar association with the C2 end
of bound purine (7). This particular interaction is apparently so
specific that a single L192I mutation in the human enzyme
resulted in a 16-fold higher apparent \( K_m \) for guanine (23). It is
not known if a similarly specific interaction exists between the
bound purine and the corresponding residue Leu-186 in Giardia
PRTase, or if the relatively high \( K_m \) for guanine in the
PRTase-catalyzed reaction could be attributed to a lack of such
specific association.

The conserved Lys residue, which interacts with the exocy-
clic O6 of bound purine through hydrogen bonding in all known
structures of purine PRTases, is also present in Giardia
PRTase as Lys-152 (Fig. 1A). However, although the e-NH2
group of Lys residue in human HGPRTase (7), T. foetus HGX-
PRTase (13) and other H/G/XPRTases (8, 9, 11) is invariably
within 3 Å from O6 of the bound purine, the corresponding
Lys-152 in Giardia PRTase is 6.3 Å away from the O6 group of bound
guanine moieties with two ordered water molecules in between.
We reasoned that this apparently much weaker bonding be-
tween the O6 of purine and Lys-152 may have provided the
basis why 6-oxopurines other than guanine cannot be sub-
strates for the PRTase-catalyzed reaction. Guanine binding
to PRTase is apparently dependent primarily on the interac-
tions of backbone carbonyls of Asp-181 and Asp-187 with the
exocyclic N2 of guanine (12). These intriguing structural features
have led us to designing, generating, and characterizing
site-specific mutants of PRTase and comparing them with the
corresponding mutants of human HGPRTase for elucidating
the roles of individual residues in the purine binding region
that determine purine specificity. In a final triple mutant
(Y127I/K152R/L186F) of the PRTase, both guanine and hy-
oxanthine can be used as substrates, indicating a successful
general increase in 6-oxopurine binding affinity in the enzyme-
catalyzed reaction.

**MATERIALS AND METHODS**

**Chemicals and Reagents**—All the chemicals used in present studies,
including hypoxanthine, guanine, xanthine, adenine, IMP, GMP, XMP,
AMP, PRPP, and the tetrasodium salt of PRPP, were purchased from
Sigma and are of the highest purity available. Xanthine oxidase (1
unit/mg of protein) was from Roche Molecular Biochemicals, and
guanine (0.1 unit/mg of protein) was from Sigma.

**Enzyme Purification**—Recombinant *G. lamblia* PRTase, cloned in a
pBace plasmid and expressed in transplanted *Escherichia coli* strain
S606 (pgb3-pro-lac, thi, hpt, rec A), was purified to homogeneity as
described previously (16). The purified enzyme samples were stored
t at \(-80{\textdegree} \text{C} \) with no detectable loss of activity after 4 months. The recomb-
inant human HGPRTase was purified from *E. coli* strain S606, transformed
with pBacpr expression vector, by a previously described procedure
(18).

**Enzyme Assays**—Kinetic data of enzyme-catalyzed reactions were
collected using a Beckman DU-640 spectrophotometer equipped with
a Peltier temperature controller. The formation of IMP and GMP were
**Equilibrium Dissociation Constant**—The dissociation constants
of GMP for binding to wild type and mutant PRTases were determined by
following the quenching of intrinsic fluorescence of Trp-180 in the
enzyme protein. Fluorescence was measured using a LS 50B fluorom-
eter (PerkinElmer Life Sciences). The excitation wavelength was
297 nm, and the emission was monitored from 300 to 400 nm. Fluores-
cence intensity was measured at different ligand concentrations, and
the fractional saturation (\( F \)) was calculated at each ligand concen-
tration. The dissociation constant was obtained using Equation 2.

\[
F = F_m\left[|L|/K_m + [L]\right]
\]

\( F_m \) is the factor allowing for maximum saturation.

**Site-directed Mutagenesis**—Site-directed mutagenesis of *G. lamblia*
PRTase and human HGPRTase genes were performed using the
Quikchange\textsuperscript{\textregistered} kit from Stratagene. Oligonucleotide primers were de-
dsigned and synthesized to generate specific mutants. Following the
polymerase chain reaction, mutant plasmid was transformed into
*E. coli* strain S606. Plasmid DNA isolated from the transformants were
sequenced for verification, and the recombinant mutant protein was
then purified from the transformed *E. coli* lysate by using the same
procedure as for the wild-type enzyme purification (16, 18). The
stability of each mutant enzyme activity was tested in repeated
assays. There was no detectable difference found between the wild
type and the mutants.

**Complementation Analysis**—Wild-type and specific site-directed mut-
ated expression plasmids were transformed into *E. coli* strain S606
that has the *de novo* synthesis enzymes and HPRTase and PRTase-
encoding genes mutated or deleted (ara, *pro-gpr-tac*, thi, hpt, purH, purF, purA, strA) (20). Cell colonies, grown on LB plates with 100 \( \mu \text{g/} \text{ml} \) ampicillin and 50 \( \mu \text{g/} \text{ml} \) streptomycin, were picked and inoculated into
LB containing both of the antibiotics. A 1% inoculum of the overnight
culture was used for growing various S60609 transformants in
the synthetic low phosphate induction medium (21) in the presence
of either 0.2 mM guanine or 0.2 mM hypoxanthine. Growth after 16 h
was monitored by light absorbance at 600 nm.

**RESULTS AND DISCUSSION**

The crystal structure of the active site in *G. lamblia*
PRTase bound to immucilin G phosphate, Mg\(^{2+}\), and PPi
(Fig. 1A) shows several apparent distinctions from, as well as
some similarities with, the human HGPRTase active site com-
plexed with GMP and Mg\(^{2+}\) (Fig. 1B). Tyr-127, stacked under
guanine in PRTase, appears to be oriented at a perpendicular
angle with respect to the bound purine ring and may not
accommodate guanine binding as well as the corresponding
Ile-135 in the human enzyme. Leu-186 in PRTase interacts
with the C2 end of the bound guanine moieties similarly to
Leu-192 in human HGPRTase. The distance between Lys-152
and the exocyclic O6 of bound guanine in PRTase is much too
far compared with the distance between Lys-165 and guanine
O6 in human HGPRTase. These residues in PRTase and the
Leu-192 residue in the human enzyme were subjected to site-
directed mutagenesis for structure-function analysis and com-
parison. Asp-187 in PRTase and Asp-193 in human HG-
PRTase are positioned in apparently equal hydrogen bonding
interactions with the exocyclic N2 of guanine. They are pre-
sumably contributing equally to guanine binding in these two
active sites and were thus not included for mutational analysis
in the present investigation.

**The Role of Tyr-127**—Sequence alignments among different
purine PRTases show a highly conserved domain, commonly
known as the PRTase fingerprint domain (Fig. 2). In this con-
served sequence, PRTase from *G. lamblia* has a Tyr at posi-
tion 127, whereas all the other purine PRTases have an Ile at
the corresponding position. In the three-dimensional structure
of PRTase, the Tyr-127 residue appears to stack below the
guanine ring with its phenolic hydroxyl group placed between
the purine exocyclic O6 and Lys-152 (Fig. 1A). A Y127I mutant
was generated, purified and characterized. The \( K_m \) value
for guanine was obtained from the individual \( K_{\text{mapest}} \) values
obtained at various PRPP concentrations by extrapolating to the
The affinity of GMP binding to *G. lamblia* GPRTase was also monitored using fluorescence quenching. Binding of GMP to the enzyme induced quenching of the intrinsic fluorescence of Trp-180 (Fig. 3), which is stacked on top of the bound guanine moiety (Fig. 1A). The dissociation constants, obtained by titration, were determined to be 36.7 ± 2.1 μM for the wild-type enzyme, whereas for the Y127I mutant it was 8.0 ± 0.7 μM. This 4–5-fold increased binding affinity of GMP to the mutant enzyme is probably responsible for the lowered *k*<sub>cat</sub> of the Y127I mutant catalyzed reaction. In the enzyme-catalyzed forward reaction, the release of GMP as the rate-limiting step has been proposed earlier in the case of human HPRTase (4) and *T. foetus* HGXPRTase-catalyzed reactions (22). The significantly higher *k*<sub>cat</sub> value of wild type *G. lamblia* GPRTase-catalyzed forward reaction not only reflects a facilitated release of GMP, but also suggests a primary *in vivo* function of the enzyme for synthesis of guanine nucleotides in *Giardia*.

![Active-site geometry in the purine binding site of *G. lamblia* GPRTase complexed with immucillin G-PO₄ and PPi (16) (A) and human HPRTase with GMP (7) (B).](image)

The Role of Leu-186—Leu-186, as seen in the active site of GPRTase (Fig. 1A), provides some apparent nonpolar interactions with the C2 end of bound guanine moiety. This particular amino acid residue is well conserved among all the purine PRTases with varied purine base specificity (7, 11, 13). Mutants of Leu-186 in *G. lamblia* GPRTase were prepared and purified as described and characterized in terms of the kinetics of catalyzed reactions. The *K<sub>m</sub>* values for guanine and the *k<sub>cat</sub>* values for catalysis in the forward reaction were determined. The kinetic constants listed in Table II indicate that the *K<sub>m</sub>* values for guanine for the L186I and the L186V-catalyzed reactions were 26- and 35-fold higher than that for the wild-type enzyme, respectively, resulting in significantly reduced catalytic efficiencies. Thus, slight changes in the side chain length and orientation of Leu-186 seem to have an overwhelmingly negative effect on the affinity of binding to guanine. A drastic shortening of the side chain also appears to have a deleterious effect on guanine binding, as observed in the 22-fold increase of *K<sub>m</sub>* for guanine in the L186A mutant-catalyzed reaction. There was, however, relatively little change in the *K<sub>m</sub>* for guanine when Leu-186 was replaced with Thr. The L186T mutant has a *K<sub>m</sub>* of 18.9 ± 1.9 μM as compared with the *K<sub>m</sub>* of 16.4 ± 1.8 μM for the wild-type, whereas there is a mere 5-fold increase in the *K<sub>m</sub>* for guanine for the L186S mutant in comparison to the wild-type enzyme. Thus, a side chain with a hydroxyl group seems to offset the significant increase in *K<sub>m</sub>* for guanine observed with other minor changes in the hydrophobic side chain of Leu-186. This discrepancy could be explained by a probable hydrogen bonding between the side chain of Thr or Ser and the exocyclic N2 and heterocyclic N3 of guanine, which would be stronger in the case of Thr compared with Ser at that particular position. Another interesting finding was that the *k<sub>cat</sub>* of L186T mutant-catalyzed reaction is only slightly lower than that of the wild type enzyme (43.0 ± 3.6 s⁻¹), thus suggesting a similar *K<sub>m</sub>* for GMP as that for the wild-type enzyme.

Another interesting observation was the *K<sub>m</sub>* of 4.9 ± 0.3 μM for guanine in the L186F catalyzed reaction. Considering the drastic decreases in the binding affinity observed even with the slightest changes in the side chain length of Leu-186, the 3-fold increase in binding affinity through a bulky phenyl group substitution in the L186F mutant represents a real surprise. It may reflect the ability of the purine-binding site in GPRTase to accommodate a very large hydrophobic group in assisting purine binding through enhanced hydrophobic interactions. Interestingly, in the structure of *T. foetus* HGXPRTase (13), there is a Phe-162 at the corresponding site in the purine binding pocket. When Phe-162 was mutated to Leu, neither the specificity nor the affinity of purine base binding changed in the mutant enzyme (22) as compared with the wild type enzyme.

The catalytic efficiencies of these mutants as per their *k<sub>cat</sub>/K<sub>m</sub>* ratios show significant drops with the L186I, L186V,
TABLE I
Comparison of kinetic constants of the GPRTase reaction of various PRTases

| Source          | $K_m$ (μM) | $k_{cat}$ (s⁻¹) |
|-----------------|------------|-----------------|
| G. lamblia      | 16.4       | 76.7            |
| T. fetus        | 2.4        | 2.5             |
| Human           | 3.6        | 9.3             |
| S. mansonii     | 3.0        | 5.7             |
| T. gondii       | 2.1        | 12.7            |

*Table II
Kinetics of Leu-186 mutants of GPRTase of G. lamblia

| Enzyme | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (s⁻¹·μM⁻¹) |
|--------|------------|-----------------|--------------------------|
| Wild type | 16.4 ± 1.8 | 76.7 ± 2.5 | 4.67                     |
| L186V   | 574.2 ± 74.9 | 6.5 ± 1.5 | 0.011                    |
| L186F   | 424.9 ± 127.2 | 43.3 ± 1.3 | 0.100                    |
| L186T   | 18.9 ± 1.9  | 430.0 ± 36.0 | 2.27                     |
| L186S   | 82.7 ± 13.3 | 11.9 ± 0.21 | 0.14                     |
| L186A   | 367.8 ± 95.4 | 11.6 ± 2.7 | 0.032                    |
| L186F   | 4.9 ± 0.3   | 10.7 ± 0.8  | 2.18                     |

and L186A mutants in comparison to the wild-type catalyzed reaction, whereas there is negligible change in the L186T and the L186F mutants. With the increased binding affinity to guanine, the relatively unchanged catalytic efficiency of L186F can be attributed to the lowered $k_{cat}$, which may reflect less efficient release of the reaction product GMP.

All the mutants listed in Table II were tested for their ability to accept hypoxanthine and xanthine as substrates, but none of them showed any improvement over the wild-type enzyme in terms of broadened purine substrate specificity (results not shown). Leu-186 is thus apparently not involved in the specificity determination of purine base binding.

Leu-192 of Human HPRTase—In human HPRTase, Leu-192 occupies the corresponding position in the purine binding pocket (7) as compared with Leu-186 in G. lamblia HPRTase. Lee et al. (23) characterized a L192T and a L192I mutant of the human HPRTase and showed its influence on purine binding in terms of the apparent $K_m$ values. There was apparently little change in the L192T mutant from the wild type, whereas the apparent $K_m$ for guanine in L192T-catalyzed reaction had an increase of about 16-fold, which is very much in agreement with our results from the L186F mutant of GPRTase. In order to characterize and compare Leu-192 in human enzyme more thoroughly with the Leu-186 in GPRTase, mutants of L192 in human HPRTase were prepared and purified and their kinetic constants determined. As shown in Table III, the L192V, L192S, and L192T mutants show negligible changes in the $K_m$ values for guanine, whereas their catalytic efficiencies show drastic decreases over the wild-type enzyme-catalyzed reaction. These human enzyme mutants appear to behave similarly to the corresponding GPRTase mutants, except for the GPRTase L186T mutant, which had a wild-type $k_{cat}/K_m$ ratio. An even more contrasting observation was made on the $K_m$ for guanine between human L192F mutant and GPRTase L186F mutant. The $K_m$ for guanine was determined to be 122.8 ± 25.6 μM for the L192F mutant, compared with 3.6 ± 0.5 μM for the wild type enzyme, representing a 35-fold increase, whereas the catalytic efficiency decreased also by 160-fold. This is the opposite from what we observed in the GPRTase L186F mutant where the $K_m$ for guanine is reduced by 3-fold from 16.4 ± 1.8 μM to 4.9 ± 0.3 μM (see Table II), and there is little change in the $k_{cat}/K_m$ ratio. Thus, the human enzyme with Phe at position 192 appears to have a negative effect on purine binding, whereas the parasite enzyme can accommodate the large Phe side chain and allow for better hydrophobic interaction with the purine base.

The Role of Lys-152—In the crystal structure of GPRTase, Lys-152 was shown to interact with exocyclic O6 over a long distance of 6.3 Å through two ordered water molecules, which would apparently constitute a rather weak interaction (12). When this Lys residue was mutated to Arg in the K152R mutant to shorten this apparent distance (Table IV), there appeared to be no major change in the $K_m$ for guanine or the $k_{cat}$ value. Hypoxanthine remained unrecognized as a substrate by the L152R mutant, suggesting that neither the increased side chain length nor the guanidino group of Arg enhanced significantly the binding of 6-oxopurines through interaction with the exocyclic O6.

In T. foetus HGXPRTase, it was shown that the conserved Lys-134, whose ε-amino group is within 3 Å of the O6 of 6-oxopurines, is the primary determinant in conferring the specificity of the enzyme toward all three 6-oxopurines: guanine, hypoxanthine, and xanthine. Mutating this Lys to either Ala or Ser in T. foetus HGXPRTase increased the $K_m$ for guanine, while...
allowing adenine to be accepted as a substrate with an estimated $K_m$ in the range of 34–54 µM (22). *G. lamblia* GPRTase shows no recognition of adenine, hypoxanthine, or xanthine as substrate (5). With the poor binding to the exocyclic O6 by Lys-152, the primary binding sites in GPRTase active site that interact with the purine moiety appear to be Leu-186 with purine C2 and the main-chain carboxyls of Asp-181 and Asp-187 with the exocyclic N2 in guanine via hydrogen bonding. It explains why only guanine is used as substrate. It also suggests that by increasing binding affinity to purine other than interacting with the exocyclic N2 may broaden the substrate specificity beyond guanine.

Furthermore, as can be seen in the crystal structure of GPRTase (Fig. 1A), the phenolic hydroxyl of Tyr-127 is placed between the e-amino group of Lys-152 and the purine O6. It probably forms a deterrent to the interaction between them and thus further weakens the power of Lys-152 in dictating 6-oxopurine binding by 6-fold (Table IV). To verify whether Arg-152 would further strengthen purine binding by the Y127I mutant through a potentially stronger hydrogen bonding with exocyclic O6, a double mutant Y127I/K152R of GPRTase was prepared, purified, and characterized. The $K_m$ for guanine was further lowered from that of the Y127I mutant and was determined to be 2.7 ± 0.3 µM (Table IV). The $k_{cat}$ value for the forward reaction remained also relatively unchanged from that of the Y127I mutant and was found to be 8.8 ± 0.4 s⁻¹. The dissociation constant for GMP was determined to be 8.8 ± 6.0 µM and a $k_{cat}$ for the forward reaction of 4.4 ± 0.7 s⁻¹ (Table IV). Thus, by improving nonspecific 6-oxopurine binding to the active site of GPRTase through stronger hydrophobic interactions via a Y127I mutation and a further strengthening of the hydrogen bonding with the exocyclic O6 by the K152R mutation, the primary dependence of purine binding on hydrogen bonding with Asp-181 and Asp-187 becomes de-emphasized. The substrate specificity becomes relaxed somewhat as a result.

### Effect of Enhanced O6 and C2 Bindings—Another attempt was made to further improve the purine binding affinity through a double mutation Y127I/L186F. The mutant was prepared and characterized kinetically. The $K_m$ for guanine was determined to be 2.8 ± 0.3 µM, 6-fold lower than that for the wild-type enzyme. The catalytic efficiency too showed an improvement by 3-fold over the wild-type enzyme catalyzed reaction is that hypoxanthine can be now used as a substrate with an estimated $K_m$ value of 54.5 ± 15.5 µM and a $k_{cat}$ for the forward reaction of 4.4 ± 0.7 s⁻¹ (Table IV). Thus, by improving nonspecific 6-oxopurine binding to the active site of GPRTase through stronger hydrophobic interactions via a Y127I mutation and a further strengthening of the hydrogen bonding with the exocyclic O6 by the K152R mutation, the primary dependence of purine binding on hydrogen bonding with Asp-181 and Asp-187 becomes de-emphasized. The substrate specificity becomes relaxed somewhat as a result.

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**TABLE III**

| Enzyme | $K_m$ (µM) | $k_{cat}$ (µM·s⁻¹) | $k_{cat}/K_m$ (µM⁻¹·s⁻¹) |
|--------|------------|---------------------|--------------------------|
| Wild type | 3.6 ± 0.5  | 9.3 ± 0.4           | 2.58                     |
| L192V | 7.1 ± 1.2 | 0.3 ± 0.02          | 0.042                    |
| L192S | 9.5 ± 1.1 | 0.02 ± 0.009        | 0.002                    |
| L192T | 8.3 ± 3.5 | 0.7 ± 0.2           | 0.084                    |
| L192F | 122.8 ± 25.6 | 2.0 ± 0.3        | 0.016                    |

**TABLE IV**

| Activity | $K_m$ (µM) | $k_{cat}$ (µM·s⁻¹) | $k_{cat}/K_m$ (µM⁻¹·s⁻¹) |
|----------|------------|---------------------|--------------------------|
| Wild type | 16.4 ± 1.8 | 76.7 ± 2.5          | 4.7                      |
| Y127I | 2.8 ± 0.2 | 4.8 ± 0.1           | 1.7                      |
| K152R | 23.3 ± 3.4 | 63.5 ± 5.5          | 2.7                      |
| Y127I/L186F | 2.7 ± 0.3 | 8.8 ± 0.4           | 3.3                      |
| Y127I/K152R/L186F | 2.7 ± 0.3 | 54.6 ± 4.8        | 20.2                     |

**TABLE V**

| Enzyme | $K_m$ (µM) |
|--------|------------|
| Wild type | 36.7 ± 2.1 |
| Y127I | 8.0 ± 0.7 |
| Y127I/K152R | 6.8 ± 0.5 |
| Y127I/L186F | 7.7 ± 0.5 |
| Y127I/K152R/L186F | 6.8 ± 0.6 |
k_{cat}/K_m ratio over the wild-type enzyme clearly indicates the complementarity of the enhanced binding obtained by three major changes in the structure of the active site in GPRTase. First, the replacement of Tyr-127 with the more conserved and less intrusive Leu; second, extending the side chain length by the K152R mutation, thus enabling enhanced interaction with the O6 of the purine moiety; third, the increased hydrophobic interactions by the presence of a Phe-186 at the base of the purine ring. The enhanced guanine binding obtained by these site-specific changes also has a supplementary effect on hypoxanthine utilization as a substrate. The triple mutant catalyzes the formation of IMP from hypoxanthine with a Km of 29.8 ± 4.1 μM and a k_{cat} of 8.0 ± 0.6 s⁻¹ (Table IV and Fig. 4). This represents a 2-fold decrease in the Km and a 3.3-fold increase in the catalytic efficiency compared with the Y172I/K152R double mutant (Table IV).

The Km values for GMP binding to the Y127I/L186F and the Y127I/K152R/L186F mutant enzymes were 7.7 ± 0.5 and 6.8 ± 0.6 μM, respectively. Thus, both the mutants showed increased binding compared to GMP as compared with the wild-type enzyme, representing about 5–6-fold increase in the binding affinity. These results clearly indicate a requirement for enhanced interactions with O6 of the purine moiety combined with strong hydrophobic binding of the purine ring for a 6-oxopurine other than guanine, such as hypoxanthine, to bind effectively to the active site of GPRTase.

Functional Complementation Analysis with E. coli Strain S6609—In order to verify the effectiveness of the triple mutant of GPRTase in converting hypoxanthine to IMP in an in vivo environment, functional complementation of the E. coli strain S6609 purine auxotroph (ara, δpro-gpt-lac, thi, hpt, pup, purH, J, strA) with the triple mutant was performed (20). DNA transformants of E. coli were grown in minimal media supplemented with specific nucleobases. Fig. 5 shows that E. coli S6609, transformed with the wild-type GPRTase expression plasmid, could grow in the presence of guanine, but showed very poor growth in the presence of hypoxanthine or without the presence of any purine base. The Y127I plasmid-transformed cells behaved quite similarly to the wild-type GPRTase transformed cells in guanine-supplemented medium. E. coli S6609 cells transfected with either the Y127I/K152R or the Y127I/K152R/L186F plasmid grew also to a similar extent as the wild type and the Y127I plasmid-transformed cells in guanine-supplemented medium.

We believe that what we have accomplished here is a major finding in our efforts to try to understand the molecular mechanism underlying the strict guanine specificity of G. lamblia GPRTase. This newly gained knowledge should also enable us to design new, potent and specific inhibitors of G. lamblia GPRTase. Guanine derivatives with an extended exocyclic O6 substituent up to a length of 6.3 Å could be developed into a potent inhibitor of this parasite enzyme while incapable of binding to the host enzyme at all.

Conclusion—The purine-binding site in G. lamblia GPRTase has a unique structural feature, which leads to the unusually restrictive purine substrate specificity of the enzyme. The weak interaction between Lys-152 and the exocyclic O6 of bound 6-oxopurine makes the apparent interaction between Asp-181, Asp-187, and the N2 group of guanine crucial for its binding. Consequently, only guanine can bind to the active site of this enzyme. By strengthening the less specific hydrophobic interactions between the purine ring and residues Leu-186 and Tyr-127, coupled with a stronger hydrogen bonding to the exocyclic O6 of purine by mutating Lys-152 to Arg, hypoxanthine can be used as substrate of the mutant enzyme. The insight gained through our intentional alteration of the purine specificity of this enzyme suggests specific routes for the design of effective anti-parasitic agents targeted to the enzyme.

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