A Single Amino Acid Substitution in the Human and a Bacterial Hypoxanthine Phosphoribosyltransferase Modulates Specificity for the Binding of Guanine[†,‡]

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ABSTRACT: Early studies involving purine salvage in *Salmonella typhimurium* resulted in the isolation and identification of a mutant strain possessing a genetically modified hypoxanthine phosphoribosyltransferase (HPRT) with enhanced substrate specificity for guanine [Benson, C. E., and Gots, J. S. (1975) *J. Bacteriol. 121*, 77−82]. To explore the molecular basis for this altered substrate specificity in the mutant *hpt* gene product, degenerate oligonucleotide primers, designed according to the N- and C-termini of the HPRT of *Escherichia coli*, were used in polymerase chain reactions to amplify both the mutant and wild-type *S. typhimurium hpt* genes from genomic DNA. Analysis of the deduced amino acid sequences revealed that a single base mutation resulted in the encoding of a Thr in the mutant HPRT, instead of an Ile found in the wild-type enzyme, at a position analogous to position 192 (Leu-192) of the human HPRT. Comparison of kinetic data for purified recombinant mutant and wild-type HPRTs showed no difference in the overall catalytic efficiency (k_{cat}/K_m) with hypoxanthine as substrate, but with guanine, the mutant enzyme exhibited a more than 50-fold higher k_{cat}/K_m largely as a result of a decrease of nearly 2 orders of magnitude in K_m. Involvement in substrate binding of the cognate amino acid at position 192 in the human HPRT was investigated using site-directed mutagenesis. Mutation of Leu-192 to Thr did not significantly alter k_{cat}/K_m values for hypoxanthine and guanine compared to wild-type, and replacement of Leu-192 with Ile had no significant change in kinetics for either hypoxanthine or PRPP. However, this Ile substitution resulted in an over 15-fold decrease in the k_{cat}/K_m for guanine due to a greater than 15-fold increase in K_m. These results demonstrate that a single active site amino acid substitution in HPRTs can significantly alter the specificity for binding guanine.

Hypoxanthine phosphoribosyltransferases[1] (HPRTs; inosine monophosphate:pyrophosphate PRTase, EC 2.4.2.7)[2] are purine salvage enzymes that catalyze the Mg^{2+}-dependent transfer of a phosphoribosyl group from phosphoribosyl pyrophosphate (PRPP) to the N9 position of 6-oxopurines (hypoxanthine, guanine, and sometimes xanthine) to form the corresponding nucleoside 5′-monophosphates IMP, GMP, or XMP, and inorganic pyrophosphate (*PPi*). The HPRTs are members of a larger family of phosphoribosyltransferases (PRTs), which are involved in *de novo* purine nucleotide biosynthetic pathways, in the salvage pathways for purine, pyrimidine, and pyridine ribonucleotide metabolism, and also in biosynthetic pathways for the aromatic amino acids histidine and tryptophan (*3*). Although relatively little primary sequence homology exists among the approximately 10 PRTs identified thus far, the crystal structures for several PRTs which have been determined to date have allowed the classification of this family of enzymes into two main types. Type I PRTs, which include HPRTs, orotate phosphoribosyltransferases (OPRTs), and glutamine phosphoribosylpyrophosphate amidotransferases (GPATs), share a common core tertiary structure, resembling a typical Rossman dinucleotide-binding fold. This common core region consists of five parallel β-strands surrounded by three or four α-helices (*4*, *5*). A short sequence of 13 amino acids present within this fold, referred to as the PRPP-binding motif, is located near the C-terminal end of the third β-strand in the core β-sheet, and is the conserved signature sequence of type I PRTs. Type II PRTs, of which quinolinic acid phosphoribosyltransferase (QAPRT) is the only member identified

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[1] In recent years, there has been a trend for investigators to include in the name of the purine phosphoribosyltransferase all of the purines salvaged by the enzyme (i.e., HPRT, HGPRT, or HGXPRT). However, many bacterial enzymes (usually referred to as HPRTs) salvage guanine in addition to hypoxanthine. Also, several enzymes referred to as HPRTs or HGPRTs (i.e., the human enzyme) salvage xanthine, albeit at low, but detectable levels (*1*, *2*). Thus, for this paper, HPRT is used in referring to all enzymes that are genetically descended from the bacterial HPRT (encoded by the *hpt* locus).

[2] Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; GPRT, guanine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; XGPRT, xanthine-guanine phosphoribosyltransferase; OPRT, orotate phosphoribosyltransferase; GPAT, glutamine phosphoribosylpyrophosphate amidotransferase; QAPRT, quinolinic acid phosphoribosyltransferase; PRPP, 5-phospho-α-D-ribosyl 1-pyrophosphate; PRT, phosphoribosyltransferase; PP_i, pyrophosphate; IMP, inosine 5′-monophosphate; GMP, guanosine 5′- monophosphate; XMP, xanthosine 5′-monophosphate; NMP, nucleoside monophosphate; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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thus far, do not possess the PRPP-binding motif or fold representative of type I PRTs, but instead possess a different tertiary structure consisting of an irregular α/β-barrel with seven β-strands and six helices (*6*).

Purine PRTs have been proposed to be potential biological targets for antiparasite chemotherapy due to the fact that parasites lack *de novo* pathways for purine nucleotide biosynthesis and thus are forced to rely on salvage pathways to replenish nucleotide pools essential for survival (*7*−*11*). Differences in substrate binding determinants between human and parasite HPRTs may be exploited in structure-based drug design of specific inhibitors of parasite HPRTs. Moreover, the study of purine salvage enzymes is biomedically important since disease states such as gouty arthritis and Lesch−Nyhan Syndrome result from amino acid mutations in the human HPRT that cause partial to complete absence of HPRT activity (*1*, *12*, *13*). For these reasons, considerable interest has focused on understanding this class of enzymes with regard to structural determinants for substrate binding and catalysis.

In contrast to most eukaryotes, which possess a single purine PRT for the salvage of 6-oxopurines, enteric bacteria possess at least two biochemically and genetically distinct enzymes for the salvage of 6-oxopurines (*14*−*17*). The HPRT is encoded by the *hpt* locus and phosphoribosylates hypoxanthine much more efficiently than guanine and has negligible activity with xanthine. The guanine phosphoribosyltransferase [GPRT (EC 2.4.2.22)] is encoded by the *gpt* locus and also is referred to as xanthine phosphoribosyltransferase (XPRT) or xanthine-guanine phosphoribosyltransferase (XGPRT). This enzyme from *Salmonella typhimurium*, *Escherichia coli*, and *Lactobacillus casei* prefers guanine and xanthine over hypoxanthine (*14*, *16*, *18*). Early genetic and biochemical analysis of deletion mutants lacking HPRTs and/or GPRTs enabled the characterization and determination of substrate cross-specificity for these two enzymes from enteric bacteria. The HPRT is responsible for 65−75% of the conversion of available hypoxanthine to IMP and 10% of the guanine to GMP. The GPRT salvages 25−35% of the hypoxanthine and 90% of the available guanine, and is the only entry point for xanthine into purine nucleotide biosynthetic pathways (*19*).

Genetic separation of the *hpt* and *gpt* genes from enteric bacteria was first accomplished in *S. typhimurium* and was facilitated by the discovery that certain *purE* mutants carrying the *proAB47* deletion possessed an additional deletion of the "*gxu*" gene (*gpt*) encoding the GPRT (*16*). These mutants were auxotrophic for purines due to both a block in the *de novo* purine biosynthetic pathway (*purE*) and the inability to utilize guanine or xanthine as the exogenous purine source due to *gxu* (*gpt*) deletion. One such strain, GP660, was subjected to chemical mutagenesis, and a derivative strain, GP36, was isolated that could utilize guanine and hypoxanthine, but not xanthine, as the exogenous purine sources. This phenotypic alteration was designated "*sug*" for "suppressor of guanine utilization" (*20*). Chromatographic and genetic analysis demonstrated that the *sug* mutation in GP36 was allelic with the *hpt* locus and thus represented a modification of the substrate specificity of the *hpt* gene product (*20*).

The availability of a mutant strain (GP36) of *S. typhimurium*, possessing an HPRT dramatically enhanced in guanine salvage, provided an opportunity to explore the molecular basis for this change in substrate specificity. In this study, we report the majority of the DNA sequence for the *hpt*s of strains GP660 and GP36, and identify a single amino acid substitution (Ile→Thr) as the mutation likely responsible for the altered substrate specificity in the mutant HPRT of GP36. To substantiate this finding, the cloned *hpt*s were overexpressed and purified to near-homogeneity, and substrate specificities were evaluated. Also, the role of the homologous amino acid in the human HPRT (Leu-192) was evaluated by the creation, expression, and kinetic analysis of L192T and L192I site-directed mutants. Herein, we report for the first time a partial amino acid sequence for the HPRT from *S. typhimurium* along with evidence supporting the participation of the amino acid at position 192 of HPRTs in influencing the binding specificity for hypoxanthine versus guanine.

MATERIALS AND METHODS

Materials. *S. typhimurium* strains GP660 (Δ*proAB-gpt*, *purE*) and GP36 (Δ*proAB-gpt*, *purE*, *sug*) were provided by Dr. Joseph Gots (University of Pennsylvania School of Medicine). The *E. coli* strain for gene expression, SØ606 (F-*ara*, Δpro-*gpt-lac*, *thi*, *hpt*, *recA*) (*21*), was obtained from Dr. Duncan Cochran (University of Queensland, Australia). Oligonucleotide primers were synthesized by the Oligonucleotide Core Facility at The University of North Carolina at Chapel Hill and Gibco BRL (Gaithersburg, MD). AmpliTaq Gold thermostable DNA polymerase was from Perkin-Elmer (Norwalk, CT). MAX Efficiency DH5α competent *E. coli* were from Life Technologies (Gaithersburg, MD). Restriction endonucleases were from New England Biolabs (Beverly, MA). [8-^{14}C]Hypoxanthine (47.1 Ci/mol) was obtained from DuPont NEN (Boston, MA). [8-^{14}C]Guanine (54.0 Ci/mol) and [8-^{14}C]xanthine (47.1 Ci/mol) were purchased from Moravek Biochemicals (Brea, CA), and [α-^{35}S]dATP for DNA sequencing was from DuPont NEN (Boston, MA). All other reagents were of the highest quality commercially available.

Amplification of the Salmonella hpt Gene Fragments by PCR. Genomic DNAs from the *S. typhimurium* GP660 and GP36 strains were extracted and purified as described (*22*). The majority of the coding regions for the wild-type (strain GP660) and mutant (strain GP36) *hpt* genes were PCR-amplified from genomic DNA using degenerate oligonucleotide primers designed according to sequence encoding the N- and C-termini of the HPRT of *E. coli* (*23*). Since amino acids 5−13 are identical in *E. coli* and *Vibrio harveyi* HPRTs, the degenerate N-terminal primer for amplification of the *Salmonella hpt* was designed beginning with the sequence encoding the Met at position 5 of the reported *E. coli* HPRT sequence (Figure 1). Unique restriction sites were incorporated into the primers to facilitate subsequent directional cloning of the PCR products. The degenerate sense primer, 5′-AAA CAT ATG AA(A/G) CA(T/C) ACN GTT-3′, contained an *Nde*I site. The degenerate antisense primer, 5′-AAA CTG CAG TTA CTC GTC CAG CAG AAT CAC TTT NCC (G/A/T)AT A-3′, contained a *Pst*I site. PCR was performed using a Perkin-Elmer 2400 thermocycler with 60 cycles of denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min, and extension at 72 °C for 2 min. Amplified PCR fragments of 552 bp were digested with *Nde*I and *Pst*I, gel-purified using the USBioclean MP Kit (USB; Cleveland,

OH), ligated into the pBAce expression vector, and cloned in *E. coli* strain DH5α. Plasmids were extracted as described (*22*) and sequenced as described below.

Analysis of Salmonella DNA Sequences. Plasmids were sequenced by the dideoxynucleotide chain termination method of Sanger et al. (*24*) using the ΔTaq Cycle Sequencing Kit from USB (Cleveland, OH). Consensus sequences of the wild-type (GP660) and mutant (GP36) *hpt*s were determined by sequencing DNA cloned from three independent PCRs. Cloned inserts were completely sequenced in the forward and reverse directions. The DNA and deduced amino acid sequences for the *S. typhimuriuum hpt*s were analyzed with the NCBI Blast sequence analysis program (*25*). Amino acid sequences for the available bacterial and the human HPRTs were aligned pairwise using the multiple sequence alignment program Clustal W (*26*). Using the program "O", version 5.10 (*27*), Thr and Ile were modeled into position 192 in the crystal structure of the human HPRT with bound GMP (*4*).

Site-Directed Mutagenesis: Creation of Mutations at Position 192 in the Human HPRT. The Leu-192 to Thr (L192T) site-directed mutation in the human HPRT was created by PCR using the method of overlap extension (*28*). The DNA template used in the mutagenesis experiment was the pBAce expression vector with the cDNA encoding the human HPRT inserted into the *Nde*I and *Sal*I restriction sites (*29*). The sequences of the sense and antisense overlapping mutagenic oligonucleotides were 5′-GTT GTA GGA TAT GCC **ACG** GAC TAT AAT GA**G** TAC TTC AGG GAT TTG-3′ and 5′-GTC **CGT** GGC ATA TCC TAC AAC AAA CTT GTC TGG AAT TTC-3′ (base changes shown in boldface type), which hybridize to bases 562−606 and 544−582, respectively, in the full-length human cDNA sequence. A *Sca*I restriction site was incorporated as a silent mutation into the sense overlapping oligonucleotide primer to facilitate subsequent identification of the desired mutant cDNA. The PCR-amplified DNA fragments were ligated into the pBAce expression plasmid and sequenced (as described above) to confirm successful creation of the L192T mutation as well as absence of secondary mutations from polymerase errors in PCR.

The Leu-192 to Ile (L192I) mutant of the human HPRT was created by PCR using the cassette mutagenesis method (*30*). The L192T human HPRT mutant in pBAce was used as template. A mutagenic antisense oligonucleotide encoding Ile at position 192 (5′-CT GAA GTA CTC ATT ATA GTC **AAT** GGC ATA TCC TAC AAC-3′) was designed according to nucleotides 562−599 in the wild-type HPRT sequence. The *Sca*I site originally created in the L192T mutant was retained in the L192I mutagenic oligonucleotide. Plasmids were sequenced to confirm the successful creation of the L192I mutation, free of polymerase errors.

Expression and Purification of HPRTs. *E. coli* strain SØ606 (*21*) was used for high-level expression of recombinant HPRTs after transformation with pBAce expression plasmids (*31*) containing the coding sequences of the GP660 or GP36 *hpt*s. Expression of recombinant HPRTs was as described previously (*32*, *33*). Cells were harvested and lysed as described (*32*). Protamine sulfate (1 mg/mL) was added to the lysate upon the final thaw, to aid in precipitation of nucleic acids (*34*). Bacterial lysates were centrifuged at 13500*g* for 30 min at 4 °C, and the recombinant HPRTs were purified to near-homogeneity from the soluble supernatants by anion exchange chromatography using a MonoQ HR-10/10 column (Pharmacia; Uppsala, Sweden). Protein was eluted by a 0−1 M NaCl gradient in TMD buffer (pH 7.5). Fractions containing purified enzyme were identified by 15% SDS−PAGE. The levels of expression of the recombinant mutant and wild-type *Salmonella* HPRTs were quantitated by densitometric analysis of the proteins resolved by 15% SDS−PAGE using a Hoefer Scientific Instruments (San Fransisco, CA) GS300 Transmittance/Reflectance Scanning Densitometer. The mutant and wild-type human HPRTs were expressed and purified as described previously (*32*, *33*).

Specific Activity Assays. HPRT activity was measured using ^{14}C-labeled hypoxanthine, guanine, or xanthine at 80 μM each and PRPP at 1 mM, employing the conditions described previously (*8*, *35*). Protein concentrations for specific activity calculations were determined using the Bio-Rad Bradford Protein Assay and bovine IgG as a standard. Radioactivity was quantitated using a Beckman LS3801 Liquid Scintillation Counter. Specific activities were calculated as described previously (*35*).

Kinetic Parameter Determinations. Steady-state values for the apparent Michaelis constant (K_m) and catalytic constant (k_{cat}) were determined spectrophotometrically as previously described (*34*, *36*). Apparent K_m values for purine bases were determined by measuring initial velocities of the forward reaction with the concentration of PRPP fixed at 400 μM. Apparent steady-state K_m values for PRPP for the forward reaction were determined with the concentration of hypoxanthine fixed at 100 μM. Assays were performed at 37 °C in a Beckman 640 spectrophotometer equipped with Peltier electronic temperature control. Steady-state apparent kinetic parameters (k_{cat} and K_m) were evaluated by direct fit of initial velocity data versus substrate concentration to the Michaelis−Menten equation using a weighted nonlinear regression method as described by Duggleby (*37*) and incorporated in the k·cat software package (Princeton, NJ).

RESULTS

Sequence Analysis of Wild-Type and Mutant HPRTs of S. typhimurium. DNA sequence comparisons of the cloned PCR-amplified wild-type (GP660) and mutant (GP36) *hpt* gene fragments from *S. typhimurium* with the full-length *hpt* from *E. coli* (*23*) suggest that approximately 90% (492 of a probable 546 nucleotides) of the *S. typhimurium hpt* genes were obtained. NCBI Blastn Search results indicate that the partial *hpt* sequence from the wild-type *S. typhimurium* is 86%, 70%, and 69% identical, at the nucleotide level, with the *hpt*s of *E. coli*, *V. harveyi*, and *Haemophilus influenza* in overlapping regions of 492, 413, and 401 bases, respectively. Alignment of the partial nucleotide sequences for the wild-type and mutant *hpt*s from the two *Salmonella* strains reveals a single base substitution encoding a Thr in the mutant HPRT where there is an Ile in the wild-type deduced amino acid sequence. The position of this mutation is homologous with position 192 (Leu-192) of the human HPRT sequence (*4*).

The deduced amino acid sequence for the wild-type HPRT of *S. typhimurium* is shown in Figure 1 aligned with the human and reported bacterial HPRT sequences. As might be expected, a high degree of sequence homology exists

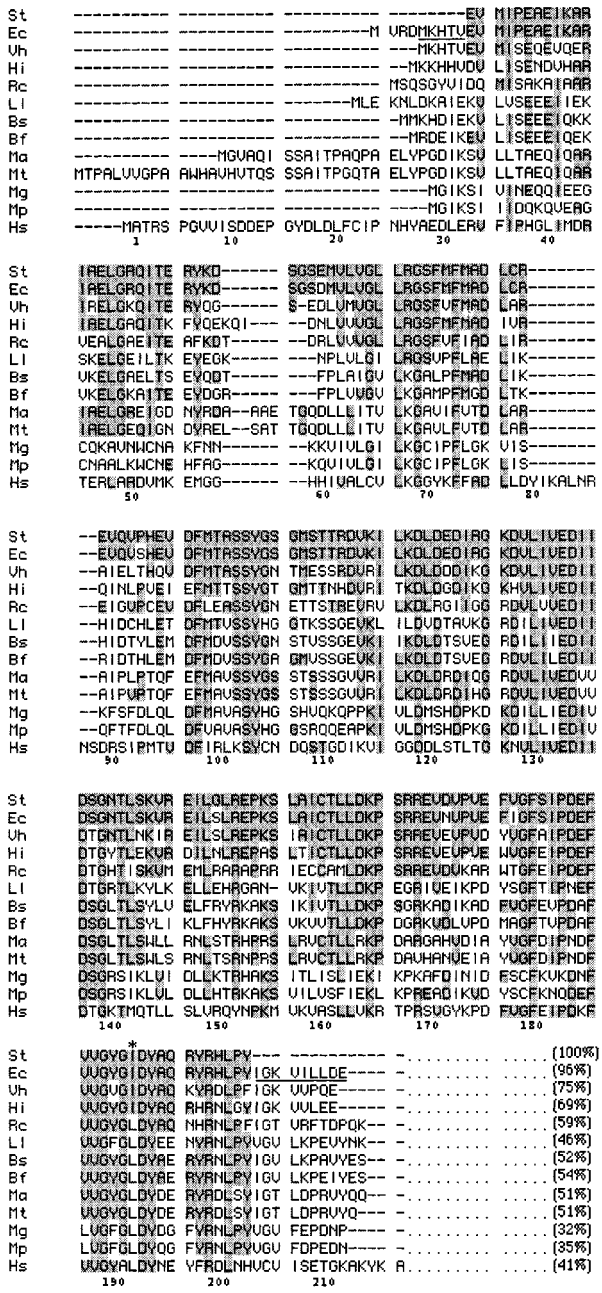

FIGURE 1: Alignment of the deduced amino acid sequence of the HPRT from *S. typhimurium* with bacterial and human HPRTs. Shading indicates exact sequence identity of amino acids with the *S. typhimurium* sequence. Numbering is according to that of the human HPRT (*4*) and is printed below the sequences. The amino acid at position 192 in the *S. typhimurium* sequence is indicated by an asterisk and is the position of the threonine mutation found in the mutant GP36 *S. typhimurium* HPRT. The N- and C-terminal regions of the HPRT from *E. coli* to which corresponding degenerate oligonucleotide primers were based for PCR amplification of the partial *hpt* from *S. typhimurium* are underlined. Alignment was performed using the multiple sequence alignment program Clustal W (*26*). Percent amino acid identities with the *Salmonella* sequence were determined by Blastp search (*25*). Numbers in parentheses at the end of each amino acid sequence (bottom right) show the percent sequence identity with the deduced *S. typhimurium* amino acid sequence. Abbreviations are as follows: *St, S. typhimurium* (this paper); *Hs, Homo sapiens* (*43*); *Ec, Escherichia coli* (*23*); *Vh, Vibrio harveyi* (*44*); *Hi, Haemophilus influenza* (*45*); *Bs, Bacillus subtilus* (*46*); *Bf, Bacillus firmus* (*47*); *Ll, Lactococcus lactis* (*48*); *Rc, Rhodobacter capsulatus* (*49*); *Mt, Mycobacterium tuberculosis* (*50*); *Ma, Mycobacterium avium* (*51*); *Mp, Mycoplasma pneumoniae* (*52*); and *Mg, Mycoplasma genitalium* (*53*).

among the bacterial HPRTs, especially in the HPRTs of the Gram-negative facultative anaerobes (*S. typhimurium*, *E. coli*, *V. harveyi*, and *H. influenza*; Figure 1). Much of the shared primary sequence identity between the human and bacterial HPRTs is confined to three short stretches corresponding to putative substrate binding regions in HPRTs (*9*, *10*, *38*, *39*) each bordered by much longer regions of relatively no sequence homology. The first region, found in almost all PRTs, is composed of 16 amino acids (residues 127−142 of the human HPRT; Figure 1) and represents the PRPP-binding motif. Five of the amino acids are identical, and nine represent conserved substitutions between the human and bacterial HPRT sequences within this region. A second conserved region, of 20 amino acids, is located at positions 182−201 of the human HPRT, and recently has been ascribed to function in purine base binding in the human HPRT crystal structure with bound GMP (*4*). Seven of these residues are identical and four conserved in the bacterial and human sequences. The Thr mutation identified herein in the mutant HPRT from *Salmonella* strain GP36 is located at position 192 within this purine-binding region. Ile and Leu are present at this position in the wild-type *Salmonella* and human HPRTs, respectively. The third short region of significant amino acid homology is located closer to the N-terminus of the aligned sequences and corresponds to residues 65−77. This region originally was predicted to be involved in binding purine bases (*9*, *38*) but has been shown to be associated with the ribose phosphate moiety of GMP in the crystal structure of the human HPRT (*4*). This motif contains three amino acid identities and six conservative substitutions among the bacterial and human HPRTs.

Inspection of the X-ray crystal structure of the human HPRT/GMP complex reveals that the side chain of Leu-192 is within van der Waals contact distance of the C2 amino group of bound GMP (*4*). Using the 3-D coordinates for the human HPRT, Thr and Ile were independently modeled into the enzyme at position 192. The three structures shown are views of the active sites of the wild-type human HPRT (Figure 2A) along with models for the Ile (Figure 2B) and Thr (Figure 2C) substitutions. The interatomic distances between the Cδ1, Cδ2, and Cβ carbons of the Leu-192 side chain in the native human enzyme and the C2 amino group of bound GMP are 4.3, 4.7, and 3.6 Å, respectively (Figure 2A). The model of Ile at position 192 reveals that the γ2 methyl group of the *sec*-butyl side chain is 2.5 Å from the C2 amino of GMP (Figure 2B). The potentially unfavorable steric interactions caused by the Ile substitution may account for the preference for hypoxanthine over guanine by the HPRTs of *S. typhimurium* and other enteric bacteria (*14*−*17*). A hydrogen at the C2 position of hypoxanthine is likely to be too small to interact with the Ile side chain. The human HPRT, however, does not discriminate significantly between hypoxanthine or guanine as substrates (*2*, *40*), which may be due to the absence of steric interference by the isobutyl side chain of Leu at position 192 with the C2 amino of GMP. The model of Thr at position 192 in the human HPRT (Figure 2C) provides a possible structural explanation for the observed increase in guanine substrate specificity by the mutant *Salmonella* HPRT (*20*). In addition to the lack of steric interference as predicted for Ile at position 192, the presence of a −OH at the β-carbon of the side chain of Thr could in theory enhance guanine binding by allowing

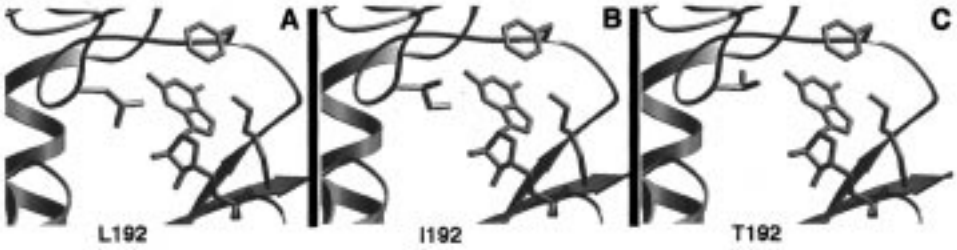

FIGURE 2: Active site views derived from the crystal structure of the human HPRT/GMP complex (*4*) showing the spatial relationship of position 192 to the C2 amino group of the GMP ligand. In Panel A is the active site of the wild-type human HPRT with Leu-192. In Panel B is the modeled active site of the human L192I mutant, and in panel C is the modeled active site of the L192T mutant.

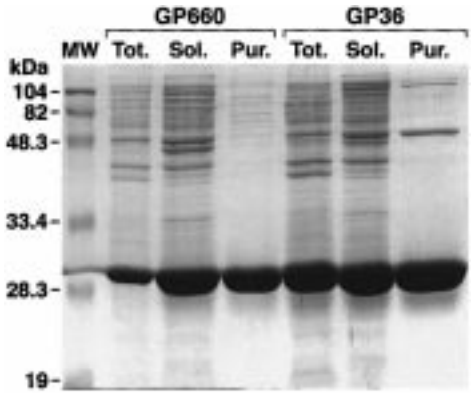

FIGURE 3: Coomassie blue-stained SDS−polyacrylamide gel showing the expression and purification of recombinant wild-type and mutant HPRTs from *S. typhimurium*. The recombinant HPRTs (GP660, wild-type; and GP36, mutant) were expressed in *E. coli* using the pBAce expression vector (*31*). The HPRTs were purified by anion exchange chromatography from the soluble fraction of the bacterial lysates. MW = molecular mass markers; Tot. = total protein in whole cell lysate; Sol. = soluble supernatant fraction of lysates; Pur. = purified enzyme.

hydrogen bonding with the C2 amino of guanine (Figure 2C).

Analysis of the Recombinant Salmonella HPRTs. To confirm involvement of the Thr mutation at position 192 in affecting substrate specificity in the mutant *S. typhimurium* HPRT, the recombinant wild-type and mutant enzymes were expressed in *E. coli* using the pBAce expression vector (Figure 3). Densitometric analysis of dried Coomassie Blue stained SDS−polyacrylamide gels demonstrated that up to 80% of the total soluble protein in the induced recombinant bacteria is of the molecular weight expected for the recombinant HPRT. The recombinant HPRTs were purified to near-homogeneity by MonoQ anion-exchange chromatography (Figure 3), and the specific activities for the forward reaction were determined with hypoxanthine, guanine, and xanthine. The recombinant wild-type GP660 HPRT exhibited nearly a 15-fold higher activity with hypoxanthine than with guanine (specific activity 102 ± 2 and 7 ± 1 μmol min^{-1} mg^{-1}, respectively) and showed negligible activity with xanthine. In contrast, the recombinant mutant GP36 HPRT possessed an approximately 2-fold higher activity with guanine than with hypoxanthine (specific activity 92 ± 2 and 40 ± 2 μmol min^{-1} mg^{-1}, respectively). The specific activity with guanine was more than 10-fold higher than for the wild-type enzyme, while that with hypoxanthine salvage was 2.5-fold lower. Although the specific activity with xanthine was determined to be higher than for the wild-type enzyme, xanthine salvage was still quite low ($>10^3$-fold lower) compared with the other substrates. These findings closely parallel those originally reported for cell-free extracts from *Salmonella* strains GP660 and GP36 (*20*), indicating that the recombinant enzymes are kinetically similar to the native enzymes.

Table 1: Steady-State Kinetic Constants with Wild-Type and Mutant Recombinant *Salmonella* and Human HPRTs

enzyme[a]	substrate[b]	app K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m
St GP660 (Ile-192)	H	5.7 ± 0.3	39	6.8
	G	370 ± 110	34	0.09
St GP36 (I192T)	H	1.8 ± 0.1	13	7.1
	G	5.6 ± 0.4	27	4.8
Hs WT (Leu-192)	H	12.1 ± 1.7	2.7	0.22
	G	9.2 ± 0.2	4.5	0.49
	PRPP (w/ H)	14.1 ± 1.5	2.2	0.15
Hs L192I	H	11.2 ± 1.7	4.0	0.36
	G	≫150	4.5	0.03
	PRPP (w/ H)	11.9 ± 0.7	4.5	0.37
Hs L192T	H	3.7 ± 0.5	1.1	0.30
	G	4.7 ± 0.8	1.6	0.30

[a] *St* = *Salmonella typhimurium*; *Hs* = *Homo sapiens*. All enzymes assayed were recombinant and purified to near-homogeneity. [b] H = hypoxanthine; G = guanine; PRPP = 5-phospho-α-D-ribosyl 1-pyrophosphate.

Table 1 summarizes the apparent steady-state kinetic parameters determined for the purified recombinant *Salmonella* HPRTs. Consistent with the results of the specific activity assays, hypoxanthine is a better substrate than guanine for the wild-type HPRT, which has more than a 75-fold greater catalytic efficiency (k_{cat}/K_m) with hypoxanthine compared to guanine. Kinetic constants for the mutant HPRT demonstrate 3-fold lower apparent K_m and k_{cat} values for hypoxanthine compared to wild-type, while the overall k_{cat}/K_m remains unchanged. With respect to guanine, although the k_{cat} for the mutant enzyme is only slightly affected, a >65-fold decrease in the apparent K_m results in more than a 50-fold increase in k_{cat}/K_m for this substrate compared to wild-type enzyme.

Site-Directed Mutations at Position 192 of Human HPRT. The Leu at position 192 of the wild-type human enzyme was mutated to Thr (the cognate residue in the mutant *Salmonella* HPRT) and to Ile (the cognate residue in the wild-type *Salmonella* HPRT). These site-directed mutants, along with the wild-type human enzyme, were expressed, purified, and assayed. The apparent steady-state kinetic parameters for the wild-type and mutant human HPRTs are shown in Table 1. The L192T mutant exhibited a >3-fold lower K_m for hypoxanthine and a >2.5-fold lower K_m for guanine. Although the k_{cat} values for both hypoxanthine and guanine were 2−3-fold lower than those for the wild-type enzyme, the catalytic efficiencies (k_{cat}/K_m) for both substrates remained largely unchanged. The L192I mutant did not significantly differ in the apparent K_m for hypoxanthine compared to the

wild-type enzyme, but the k_{cat} was slightly increased. Although initial velocities for reactions with guanine were linear to 300 μM, substrate precipitation at higher concentrations in the reaction buffer prevented the determination of velocities at saturating levels with the L192I mutant. Thus, the apparent K_m of guanine was estimated to be significantly above 150 μM. Based upon the maximal velocity observed, a minimal k_{cat} was determined to be 4.5 s^{-1} which allowed an approximation of k_{cat}/K_m. This value was over 15-fold lower than that for the wild-type enzyme with guanine (caused primarily by the increase in K_m), indicating that replacement of Leu-192 by an Ile in the human HPRT adversely affects the ability of the enzyme to use guanine as a substrate without significantly affecting the salvage of hypoxanthine.

To rule out effects of mutations at position 192 in affecting binding, catalytic rate, and catalytic efficiency for PRPP, the apparent steady-state kinetic parameters for PRPP were determined spectrophotometrically for the wild-type human and L192I mutant enzymes at a saturating concentration (100 μM) of hypoxanthine (Table 1). No significant difference was observed in PRPP binding affinities (K_m) between the wild-type and L192I mutant. However, a 2-fold higher k_{cat} and k_{cat}/K_m for PRPP was observed in the L192I mutant compared to wild-type. These findings suggest that the effect of mutation at position 192 largely involves purine base binding and specificity for hypoxanthine and guanine with only minor effects on the kinetics for PRPP.

DISCUSSION

Crystal structures of several purine PRTs have been used formerly to identify active site amino acid residues involved in conferring substrate specificity among 6-oxopurines (*4*, *5*, *41*, *42*). Three types of interactions have been considered in these reports: (A) an interaction between the 6-oxo group of the purine base and the side chain of a positively charged lysine residue (Lys-134 in *Tritrichomonas foetus* HPRT, Lys-178 in *Toxoplasma gondii* HPRT, Lys-165 in human HPRT, and Lys-115 in *E. coli* GPRT; note: these lysine residues are located in homologous positions in the 3-D structures and are completely conserved in alignments of available HPRT sequences, and this interaction likely discriminates against binding of 6-aminopurines such as adenine); (B) a $\pi-\pi$ stacking interaction between the purine base and an aromatic side chain of the enzyme (Phe-162 in *T. foetus* HPRT, Trp-199 in *T. gondii* HPRT, Phe-186 in human HPRT, and Trp-134 in *E. coli* GPRT); and (C) a less well-defined interaction that dictates specificity for binding of hypoxanthine, guanine, or xanthine, all of which differ from one another only in their 2-position substituents.

The human HPRT/GMP crystal structure (*4*) provides evidence for the main chain carbonyl oxygens of Val-187 and Asp-193 forming hydrogen bonds with the 2-amino group of guanine in GMP. Either a hydrogen or a carbonyl oxygen at the 2-position of hypoxanthine or xanthine, respectively, could be accommodated in the purine-binding pocket, but the crystal structure does not provide an explanation for the poor activity with xanthine by the human HPRT. In the *T. foetus* HPRT (*41*), the −OH group on the side chain of Tyr-156 was proposed as a candidate for forming hydrogen bonds with the 2-position moieties of guanine or xanthine. With regard to the *T. gondii* HPRT (*42*), although specific active site residues were not identified as being involved in discriminatory interactions with the exocyclic 2-position of 6-oxopurines, the authors suggested that subtle backbone interactions may be responsible. The structural data from the *E. coli* GPRT (*5*) provide evidence for the involvement of Gln-137 in discrimination among 6-oxopurine substrates via side and main chain interactions with 2-position substituents. In summary, a variety of amino acid residues in HPRTs have been proposed to participate in discrimination among 6-oxopurine substrates, but these residues are not located in homologous positions in the crystal structures for these enzymes.

Position 192, or its homologue in various HPRT crystal structures, previously has not been identified to play a role in determining substrate specificity for binding 6-oxopurines. The results of genetic, biochemical, and molecular modeling studies reported here demonstrate that this active site residue is near enough to the C2-position of 6-oxopurines to influence the specificity for substrate binding in both the human and *Salmonella* HPRTs. This hydrophobic residue would have been difficult to predict as a determinant of substrate specificity were it not for the earlier identification and isolation of a strain of *S. typhimurium* (GP36) possessing a mutant HPRT with enhanced substrate specificity for guanine (*20*). Examination of available primary HPRT sequences from eukaryotes (*9*, *10*, *39*) and prokaryotes (Figure 1) indicates conservation of hydrophobic residues at position 192. All available bacterial HPRT sequences have either Ile or Leu at position 192 (Figure 1). Furthermore, HPRTs with Ile at this position are all from bacteria which are Gram-negative facultative anaerobes (*S. typhimurium*, *E. coli*, *V. harveyi*, and *H. influenza*; Figure 1), all of which have been shown to be much more efficient in salvaging hypoxanthine than guanine (this paper and NCBI protein database). Although most of the other bacterial HPRTs have not been fully characterized with regard to substrate specificity, NCBI protein database entries for the HPRTs of *Bacillus subtilus*, *Lactococcus lactus*, and *Rhodobacter capsulatus* indicate that guanine can replace hypoxanthine as a substrate. HPRTs from these three bacteria have Leu residues at position 192 in their deduced amino acid sequences (Figure 1). Thus, for those bacterial HPRTs which have been at least partially characterized, those with Leu at position 192 appear to efficiently salvage both guanine and hypoxanthine and seem to be present in bacteria in which a separate gene coding for a GPRT or XPRT has not been reported. For *Salmonella*, and perhaps other enteric bacteria, the presence of Ile at position 192 may reduce the efficiency for guanine salvage due to unfavorable steric interactions between the γ2 methyl group on the Ile side chain and the C2 amino moiety of guanine. As a consequence, it may be more adaptive for these enteric bacteria to have an additional purine PRT (i.e., GPRT) for the efficient salvage of guanine and xanthine.

Examination of HPRT sequences from parasites (*9*, *10*) reveals that the *Schistosoma mansoni*, *Plasmodium falciparum*, and *Trypanosoma cruzi* HPRTs all have a Leu at position 192 similar to the human enzyme. In these organisms and in humans, the HPRTs are thought to be the only purine phosphoribosyltransferases present for 6-oxopurine salvage. No evidence for a separate GPRT or XPRT

exists. *Toxoplasma gondii*, *Tritrichomonas foetus*, *Leishmania donovani*, and *Crithidia fasciculata* HPRT sequences deviate in the amino acid residue found at position 192 in that they have Tyr, Phe, Met, and Met, respectively, at this position. In these organisms whose HPRTs do not possess Leu or Ile at position 192, it is of interest to note that the *T. gondii* and *T. foetus* enzymes are among the only HPRTs that are quite proficient in xanthine salvage with K_m values only 10-fold higher than for hypoxanthine and guanine (*54*, *55*). The involvement of these amino acids in dictating xanthine binding is difficult to explain and suggests that residues other than 192 may contribute to xanthine specificity in these HPRTs. Further structural and biochemical studies are required to explore these interactions. In *L. donovani*, whose HPRT possesses Met instead of Leu or Ile at position 192, evidence exists of a separate enzyme for xanthine salvage (*56*, *57*). Initial studies of purine PRTs of *C. fasciculata*, the other parasite with Met at position 192 in its HPRT, suggest separate purine PRTs for hypoxanthine and guanine salvage may be present in this organism, but xanthine salvage for either of these PRTs has not been determined (*58*). These findings are consistent with our results implicating involvement of position 192 in influencing 6-oxopurine substrate specificity in HPRTs including the possible relationship between the substituent at this position and the multiplicity of enzymes for 6-oxopurine salvage. These subtle differences in binding sites at position 192 potentially could be exploited in the future design of inhibitors selective for the HPRTs of parasites.

Although position 192 of HPRTs had not formerly been identified as playing a role in determining the specificity for binding 6-oxopurines, the results of the present study show that this single active site residue is clearly responsible for the definitive biochemical difference between the human and *Salmonella* HPRTs with respect to their efficiencies in guanine salvage. This discovery could not have been predicted from available 3-D structures or biochemical data and emphasizes the importance and potential utility of employing traditional biochemical and genetic procedures to complement contemporary approaches for investigating protein structure−function relationships.

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REFERENCES

1. Kelley, W., Rosenbloom, F., Henderson, J., and Seegmiller, J. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 1735−1739.
2. Krenitsky, T. A., Papaioannou, R., and Elion, G. B. (1969) *J. Biol. Chem. 244*, 1263−1270.
3. Musick, D. L. (1981) *CRC Crit. Rev. Biochem. 11*, 1−34.
4. Eads, J. C., Scapin, G., Xu, Y., Grubmeyer, C., and Sacchettini, J. C. (1994) *Cell 78*, 325−334.
5. Vos, S., de Jersey, J., and Martin, J. L. (1997) *Biochemistry 36*, 4125−4134.
6. Eads, J. C., Ozturk, D., Wexler T. B., Grubmeyer, C. and Sacchettini, J. C. (1997) *Structure 5*, 47−58.
7. Senft, A. W., and Crabtree, G. W. (1983) *Pharmacol. Ther. 20*, 341−356.
8. Dovey, H. F., McKerrow, J. H., and Wang, C. C. (1984) *Mol. Biochem. Parasitol. 11*, 157−167.
9. Craig, S. P., III, McKerrow, J. H., Newport, G. R., and Wang, C. C. (1988) *Nucleic Acids Res. 16*, 7087−7101.
10. Ullman, B., and Carter, D. (1995) *Infect. Agents Dis. 4*, 29−40.
11. Craig, S. P., III, and Eakin, A. E. (1997) *Parasitol. Today 13*, 238−241.
12. Lesch, M., and Nyhan, W. (1964) *Am. J. Med. 36*, 561−570.
13. Seegmiller, J., Rosenbloom, F., and Kelly, W. (1967) *Science 155*, 1682−1684.
14. Krenitsky, T. A., Neil, S. M., and Miller, R. L. (1970) *J. Biol. Chem. 245* (10), 2605−2611.
15. Chou, J. Y., and Martin, R. G. (1972) *J. Bacteriol. 112* (2), 1010−1013.
16. Gots, J. S., Benson, C. E., and Shumas, S. R. (1972) *J. Bacteriol. 112* (2), 910−916.
17. O'Reilly, C. O., Turner, P. D., Smith-Keary, P. F., and McConnell, D. J. (1984) *Mol. Gen. Genet. 196*, 152−157.
18. Miller, R. L., Ramsey, G. A., Krenitsky, T. A., and Elion, G. B. (1972) *Biochemistry 11*, 4723−4731.
19. Gots, J. S., and Benson, C. E. (1973) in *Purine Metabolism in Man* (Sperling, O., DeVries, A., and Wyngaarden J. B., Eds.) pp 33−39, Plenum Publishing Corp., New York.
20. Benson, C. E., and Gots, J. S. (1975) *J. Bacteriol. 121* (1), 77−82.
21. Jochimsen, B., Nygaard, P., and Vestergaard, T. (1975) *Mol. Gen. Genet. 143*, 85−91.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor University Press, Cold Spring Harbor, NY.
23. Fujita, N., Mori, H., Yura, T., and Ishihama, A. (1994) *Nucleic Acids Res. 22*, 1637−1639.
24. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
25. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403−410.
26. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res. 22*, 4673−4680.
27. Jones, T. A., Zhou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. A47*, 110−119.
28. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 51−59.
29. Kanaaneh, J., Craig, S. P., III, and Wang, C. C. (1994) *Eur. J. Biochem. 223* (2), 595−601.
30. Vallette, F., Mege, E., Reiss, A., and Adesnik, M. (1989) *Nucleic Acids Res. 17* (2), 723−733.
31. Craig, S. P., III, Yuan, L., Kuntz, D. A., McKerrow, J. H., and Wang, C. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2500−2504.
32. Eakin, A. E., Guerra, A., Focia, P. J., Torres-Martinez, J., and Craig, S. P., III (1997) *Antimicrob. Agents Chemother. 41*, 1686−1692.
33. Craig, S. P., III, Focia, P. J., and Fletterick, R. J. (1997) *Biochim. Biophys. Acta 1339*, 1−3.
34. Yuan, L., Craig, S. P., III, McKerrow, J. H., and Wang, C. C. (1992) *Biochemistry 31*, 806−810.
35. Yuan, L., Craig, S. P., III, McKerrow, J. H., and Wang, C. C. (1990) *J. Biol. Chem. 23*, 13528−13532.
36. Giacomello, A., and Salerno, C. (1978) *J. Biol. Chem. 253*, 6038−6044.
37. Duggleby, R. G. (1981) *Anal. Biochem. 110*, 9−18.
38. Hershey, H. V., and Taylor, M. W. (1986) *Gene 43*, 287−293.
39. Allen, T. E., and Ullman, B. (1993) *Nucleic Acids Res. 21*, 5431−5438.
40. Xu, Y., Eads, J., Sacchettini, J. C., and Grubmeyer, C. (1997) *Biochemistry 36*, 3700−3712.
41. Somoza, J. R., Chin, M. S., Focia, P. J., Wang, C. C., and Fletterick, R. J. (1996) *Biochemistry 35*, 7032−7040.
42. Schumacher, M. A., Carter, D., Roos, D. S., Ullman, B., and Brennan, R. G. (1996) *Nat. Struct. Biol. 3*, 881−887.

43. Jolly, D. J., Okayama, H., Berg, P., Esty, A. C., Filpula, D., Bohlen, P., Johnson, G. G., Shively, J. E., Hunkapillar, T., and Friedmann, T. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 477−481.
44. Showalter, R. E., and Silverman, M. R. (1990) *Nucleic Acids Res. 18*, 4621.
45. Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J.-F., Dougherty, B. A., Merrick, J. M., McKenney, K., Sutton, G., Fitzhugh, W., Fields, C. A., Gocayne, J. D., Scott, J. D., Shirley, R., Liu, L.-I., Glodek, A., Kelley, J. M., Weidman, J. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghagen, N. S. M., Gnehm, C. L., McDonald, L. A., Small, K. V., Fraser, C. M., Smith, H. O., and Venter, J. C. (1995) *Science 269*, 496−512.
46. Ogasawara, N., Nakai, S., and Yoshikawa, H. (1994) *DNA Res. 1*, 1−14.
47. Ito, M., and Krulwich, T. A. (1996) GenBank Accession No. *U61844*. *Biochemistry*.
48. Nilsson, D., and Lauridsen, A. A. (1992) *Mol. Gen. Genet. 235*, 359−364.
49. Beckman, D. L., and Kranz, R. G. (1991) *Biochim. Biophys. Acta 1129*, 112−114.
50. Philipp, W. J., Poulet, S., Eiglmeier, K., Pascopella, L., Balasubramanian, V., Heym, B., Bergh, S., Bloom, B. R., Jacobs, W. R., and Cole, S. T. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 3132−3137.
51. Bergson, S. E., Barry, R., Allen, T., Hwang, H., and Ullman, B. Unpublished. NCBI Seq ID: 1864151.
52. Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B. C., and Herrmann, R. (1996) *Nucleic Acids Res. 24*, 4420−4449.
53. Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., Bult, C. J., Kerlavage, A. R., Sutton, G., Kelley, J. M., Fritchman, J. L., Weidman, J. F., Small, K. V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, J. F., Saudek, D. M., Phillips, C. A., Merrick, J. M., Tomb, J. F., Dougherty, B. A., Bott, K. F., Hu, P. C., Lucier, T. S., Peterson, S. N., Smith, H. O., Hutchison, C. A., III, and Venter, J. C. (1995) *Science 270*, 397−403.
54. Beck, J. T., and Wang, C. C. (1993) *Mol. Biochem. Parasitol. 60*, 187−194.
55. Donald, R. G. K., Carter, D., Ullman, B., and Roos, D. S. (1996) *J. Biol. Chem. 271*, 14010−14019.
56. Tuttle, J. V., and Krenitsky, T. A. (1979) *J. Biol. Chem. 255*, 909−916.
57. Hwang, H. Y., and Ullman, B. (1997) *J. Biol. Chem. 272*, 19488−19496.
58. Kidder, G. W., Nolan, L. L., and Dewey, V. C. (1979) *J. Parasitol. 65* (4), 520−525.
59. Evans, S. V. (1993) *J. Mol. Graphics 11*, 134−138.

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