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Tinkering with enzymes*

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Abstract

Hypoxanthine guanine phosphoribosyltransferase (HGPRT) catalyses the phosphoribosylation of hypoxanthine and guanine by transferring the phosphoribosyl moiety from phosphoribosylpyrophosphate (PRPP) onto N9 in the purine base resulting in the formation of inosine monophosphate (IMP) and guanosine monophosphate (GMP). Human HGPRT can phosphoribosylate only hypoxanthine and guanine while the enzyme of the protozoan parasite, *Plasmodium falciparum*, has an additional substrate specificity for xanthine. Our aim has been to elucidate structural features in HGPRT that govern substrate specificity. We have addressed this problem by engineering chimeric HGPRTs which contain domains from both the parasite and human enzymes. The chimeric enzyme DS1 in which the first 49 residues of human HGPRT were replaced with the corresponding residues from the *P. falciparum* enzyme exhibited additional specificity for xanthine. A rational for this additional specificity for xanthine is proposed using the available crystal structures of human HGPRT and *Salmonella* orotate phosphoribosyltransferase (OPRT).

Keywords: Plasmodium, malaria, hypoxanthine–guanine phosphoribosyltransferase, protein engineering.

1. Introduction

Most reactions in living systems are catalysed by a class of proteins known as enzymes. Catalysis by enzymes is carried out at very rapid rates, with remarkably high degree of specificity and most often at the ambient temperature of 37°C. This paper addresses the question of substrate specificity exhibited by enzymes and tries to unravel structural features that govern substrate specificity in hypoxanthine–guanine phosphoribosyltransferase (HGPRT).

HGPRT, an enzyme in the purine salvage pathway, catalyses the transfer of a phosphoribosyl moiety from phosphoribosylpyrophosphate (PRPP) to N9 of hypoxanthine or guanine.[1] Human HGPRT phosphoribosylates the purine bases hypoxanthine and guanine while the enzyme from parasitic protozoa exhibits variation in substrate specificity[2] (Table I). HGPRT from *Plasmodium falciparum*,[3] *Tritrichomonas foetus*,[4] *Toxoplasma gondii*[5] and *Eimeria tenella*[6] have an additional specificity for xanthine apart from hypoxanthine and guanine. However, the enzyme from *Giardia lamblia* can catalyse the phosphoribosylation of only guanine.[7] The prokaryote, *E. coli*, has HPRT and GXPRT activities residing in two different enzymes.[8]

The two HGPRTs that we have been utilising to address the question of hypoxanthine/guanine/xanthine specificity are those from human and *P. falciparum*. The amino acid sequences of human[9] and *P. falciparum* HGXPRT[10–12] (PfHGXPRT) exhibit 78% homology

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Table I
Substrate specificities of human and parasitic protozoan HG(X)PRTs

Organism	Substrate[a]			K_m(μM)		
				H	G	X
Human[18]	H	G		0.45	3.5	
Trypanosoma brucei[2]	H	G	Allopurinol	2.3	4.8	
Trypanosoma cruzi[2]	H	G	Allopurinol	6.4	9.9	
Leishmania donovani[2]	H	G	Allopurinol	6.1	5.1	
Crithidia fasciculata[2]	H	G		NA	NA[b]	
Tritrichomonas foetus[2]	H	G	X	4.1	3.8	52
Plasmodium falciparum[17,3]	H	G	X	0.9 (0.4)	1.4(0.3)	420(29)[c]
Toxoplasma gondii[20]	H	G	X	1.69	1.01	17.6
Eimeria tenella[2]	H	G	X	0.25	0.37	15
Giardia lamblia[7]		G		514	29.5	543

[a]H: Hypoxanthine, G: Guanine and X: Xanthine
[b]NA: Data not available
[c]Numbers in parentheses correspond to K_m values obtained using partially purified HGXPRT from *P. falciparum*.[3] Numbers outside parentheses were obtained using highly purified recombinant *P. falciparum* HGXPRT.[17] There is a significant difference in the K_m for xanthine between the native parasite-derived enzyme and the recombinant *P. falciparum* HGXPRT.

(Fig. 1). The strategy adopted for this study involves construction of chimeric HGPRTs consisting of domains from *P. falciparum* and human enzymes.

Four chimeric HGPRTs were constructed and expressed in *E. coli*. One of them, DS1, a human HGPRT with 49 residues replaced by the corresponding PfHGXPRT sequence, was found to have acquired xanthine specificity apart from its original affinity for hypoxanthine and guanine. This paper presents preliminary results obtained on the chimeric HGPRTs.

2. Materials and methods

Restriction enzymes, Taq DNA polymerase, T4 DNA ligase and other molecular biology reagents were purchased from Amersham Pharmacia, UK, and Bangalore Genei Pvt Ltd, India. Sϕ609[13] was a gift from Dr Per Nygaard. PRPP, purine bases and other molecular biology reagents were from Sigma Chemical Company, USA.

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HPRT_PLAFG   MPIPNNPGAGENAFDPVFVNDDD-GYDLDSFMIPAHYKKYLTKVLVPNGVIKNRIEKLAY
HPRT_HUMAN   --ATRSP------G---VVISDDEPGYDLDLFCIPNHYAEDLERVFIPHGLIMDRTERLAR
                    *          *   **  ***** * ** **   *  *  * * *  * * **
HPRT_PLAFG   DIKKVYNNEEFHILCLLKGSRGFFTALLKHLSRIHNYSAVETSKPLFGEHYVRVKSYCND
HPRT_HUMAN   DVMKEMGGHHIVALCVLKGGYKFFADLLDYIKALNRNS--DRSIPMT-VDFIRLKSYCND
             *  *            ** ***   **  **          *    * *           * ******
HPRT_PLAFG   QSTGTLEIVS-EDLSCLKGKHVLIVEDIIDTGKTLVKFCEYLKKFEIKTVAIACLFIKRT
HPRT_HUMAN   QSTGDIKVIGGDDLSTLTGKNVLIVEDIIDTGKTMQTLLSLVRQYNPKMVKVASLLVKRT
             ****         *** * ** ************              * *  * *  ***
HPRT_PLAFG   PLWNGFKADFVGFSIPDHFVVGYSLDYNEIFRDLDHCCLVNDEGKKKYKATSL
HPRT_HUMAN   PRSVGYKPDFVGFEIPDKFVVGYALDYNEYFRDLNHVCVISETGKAKYKA---
             *   * * ***** *** ***** ***** **** * *      ** ****
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FIG. 1. Amino acid sequence alignment of human and *P. falciparum* HG(X)PRT using CLUSTALW.[26] Identical amino acids are marked with asterisk.

2.1. *Construction of domain switches*

The human HGPRT and PfHGXPRT genes were cloned into the expression vector pTrc99A[14] to obtain the plasmids pGPRT and pFPRT (Figs 2a and b). Schematic representation of the construction of domain switches 1-4 is illustrated in Figs 2a and b. To obtain domain switch 1 (DS1) (Fig. 2a), pGPRT was cleaved with NcoI and XhoI to remove the first 49 amino acid residues. The corresponding region in the PfHGXPRT (58 amino acids) was amplified by PCR

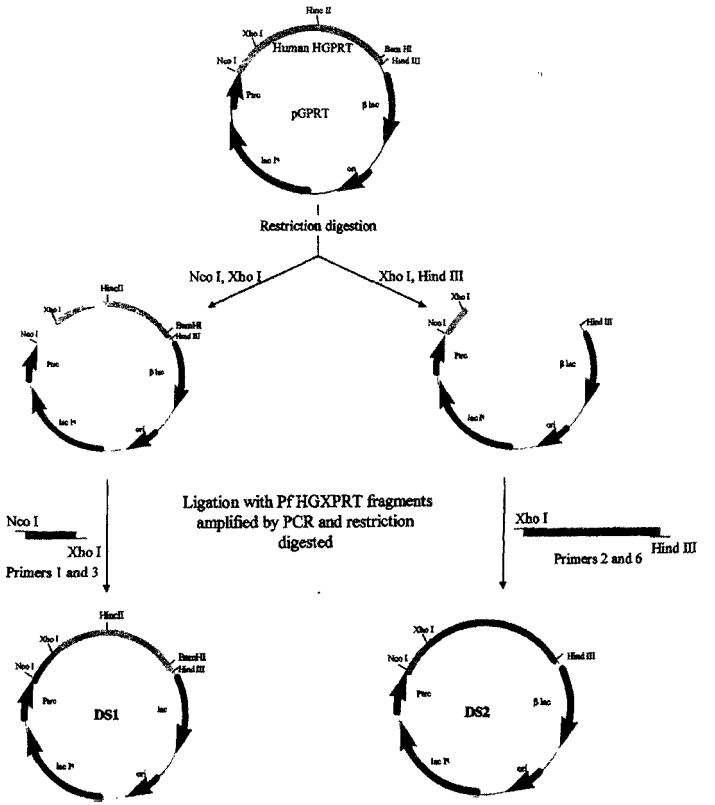

FIG. 2a. Schematic representation of the construction of the chimeras DS1 and DS2.

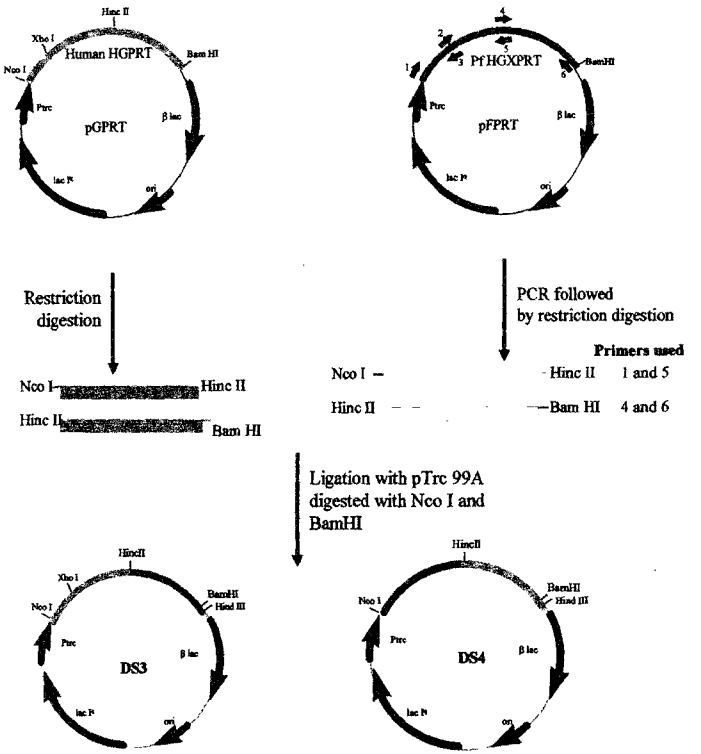

FIG. 2b. Schematic representation of the construction of the chimeras DS3 and DS4. See section on Materials and methods for primer sequences.

using primer 1(5'CCACCATGGCAATACCAAATAATCC3') and primer 3(5'AATATC-TCGAGCCAATTTCTCAATAC3') and ligated with NcoI–XhoI-digested pGPRT. Domain switch 2 (Fig. 2a) was obtained by removing the XhoI–HindIII fragment from pGPRT followed by ligation with the corresponding PfHGXPRT gene fragment amplified by PCR using primer 2(5'TTGGCTCGAGATATTAAAAAGG3') and primer 6(5'CCAAAGCTTTT-ATAATGAAGTTGCTTTAT3'). Domain switches 3 and 4 were made as shown in Fig. 2b. The primers used for DS3 were primer 4(5'GACCAGTCAACAGGTACAT3') and primer

6(5'CCAGGATTCTTATAATGAAGTTGCTTTAT3'). Primers for DS4 were primer 1(5'CCACCATGGCAATACCAAATAATCC3') and primer 5(5'CTGTTGACTGGTCATTAC3'). *E. coli* strain Sϕ609 (*ara*, Δ*pro-gpt-lac*, *thi*, *hpt*, *pup*, *purH*, *J*, *strA*) transformed with the various constructs was used for complementation studies and hyperexpression of the recombinant enzyme. The wild-type and chimeric HGPRT genes were sequenced using an automated DNA sequencer .

2.2. *Expression of wild-type and chimeric HGPRTs*

Sϕ609 *E. coli* cells carrying expression plasmids were allowed to grow overnight at 37°C in LB medium containing ampicillin (100μg/ml) and streptomycin (25μg/ml). Terrific broth containing ampicillin and streptomycin was inoculated with 1% of the overnight culture and the cells allowed to grow till A_{600} of 0.6 was reached. The cultures were then induced with 1mM IPTG and allowed to grow for a further 24 h at 37°C. Uninduced cells, also grown for 24 h, were used as controls. The cells were analysed for expression of recombinant HGPRT by SDS–PAGE, stained with coomassie brilliant blue R250.

2.3. *Functional complementation*

Complementation studies were carried out using the *E. coli* strain Sϕ609 (*ara*, Δ*pro-gpt-lac*, *thi*, *hpt*, *pup*, *purH*, *J*, *strA*) transformed with the expression plasmid constructs. The cells grown overnight in LB medium with ampicillin and streptomycin were washed with and resuspended in 1X M9 salt solution. These cells were inoculated into minimal medium containing 1X M9 salts, 1mM $MgSO_4$, 0.1 mM $CaCl_2$, 1mM thiamine hydrochloride, 1mM proline, 0.2% glucose, 0.3mM IPTG, 25μg/ml streptomycin, 100μg/ml ampicillin and 0.5 mM hypoxanthine/guanine/xanthine. The cells were allowed to grow for 15 h at 37°C and A_{600} was recorded.

2.4. *Assay for HGPRT activity*

Cells pelleted from 25 ml of induced cultures were resuspended in buffer containing 50mM Tris.Cl, pH 7.5, 2mM $MgCl_2$, 10% glycerol, 100μg/ml PMSF, 3mM β-mercaptoethanol and sonicated. The crude lysates were centrifuged at 13,000 g for 15 min and the supernatant assayed for enzyme activity. The assay mixture consisted of 100mM Tris.Cl pH 7.4, 12mM $MgCl_2$, 1mM PRPP and 50μM of the purine base (hypoxanthine/guanine/xanthine). Phosphoribosyltransferase activity was assayed spectrophotometrically by measuring increase in absorbance at 245, 257.5 and 255 nm for hypoxanthine, guanine and xanthine, respectively. The $\Delta\varepsilon$ valves used were 1900 $M^{-1}cm^{-1}$ (hypoxanthine $\rightarrow$ IMP), 5900$M^{-1}cm^{-1}$ (guanine $\rightarrow$ GMP) and 4685$M^{-1}cm^{-1}$ (xanthine $\rightarrow$ XMP).[7, 15, 16, 21]

3. Results

Construction of the chimeric HGPRT genes DS 1–4 is shown in Fig. 2. Human HGPRT gene fragments required for the construction of DS 1–4 were generated by restriction digestion of the wild-type gene with appropriate restriction enzymes. PCR was used to generate the required PfHGXPRT fragments and the sequences of the primers used in the amplification are

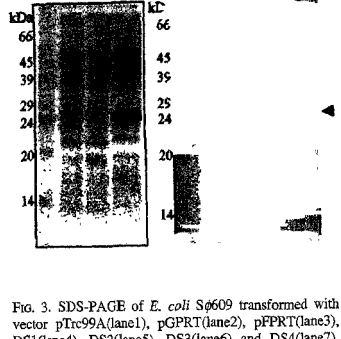

FIG. 3. SDS-PAGE of *E. coli* Sϕ609 transformed with vector pTrc99A(lane1), pGPRT(lane2), pFPRT(lane3), DS1(lane4), DS2(lane5), DS3(lane6) and DS4(lane7). The cell were induced with 1mM IPTG for 24 h. Bands of expected molecular weight were seen only in the case of pFPRT, DS1 and DS4. Hyperexpressed protein is indicated by an arrow.

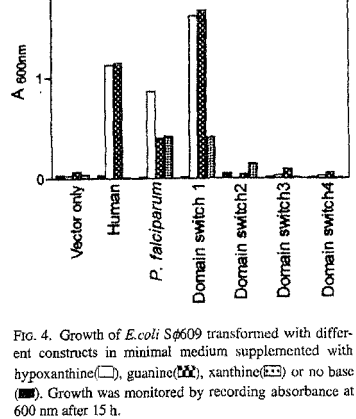

FIG. 4. Growth of *E.coli* Sϕ609 transformed with different constructs in minimal medium supplemented with hypoxanthine(□), guanine(▨), xanthine(▤) or no base (■). Growth was monitored by recording absorbance at 600 nm after 15 h.

indicated in Materials and methods. All wild-type and chimeric HGPRTs were completely sequenced and found to be devoid of PCR errors. The wild-type and chimeric HGPRTs were cloned into the *E. coli* expression vector pTrc99A so as to produce unfused recombinant enzyme upon induction with IPTG.

SDS–PAGE analysis of IPTG-induced clones was carried out to ascertain levels of recombinant enzyme expression (Fig. 3). PfHGXPRT, DS1 and DS4 exhibited high levels of expression. However, no induced band could be seen on SDS–PAGE upon induction with IPTG of the human HGPRT, DS2 and DS3 clones in Sϕ609 (Fig. 3).

3.1. *Functional complementation*

The expression constructs of wild-type and chimeric HGPRTs were routinely analysed for production of functionally active enzyme by monitoring their ability to complement hypoxanthine/guanine/xanthine PRT deficiency in the mutant strain Sϕ609 (*ara*, Δ*pro-gpt-lac*, *thi*, *hpt*, *pup*, *purH*, *J*, *strA*). In Sϕ609, human and PfHGXPRT expressing clones display the expected activities (Fig. 4). Of the 4 chimeras, only DS1 was able to complement HGXPRT deficiency in Sϕ609. DS2, 3 and 4 were inactive. Interestingly, chimera DS1, apart from retaining hypoxanthine and guanine activities of human HGPRT had acquired an additional specificity for xanthine (Fig. 4).

HGPRT activity in crude lysates was measured by monitoring increase in absorbance at 245 nm for hypoxanthine, 257.5 nm for guanine and 255 nm for xanthine. Figure 5 summarizes the assay results of wild-type and chimeric HGPRTs. The activity profiles agree with the complementation data.

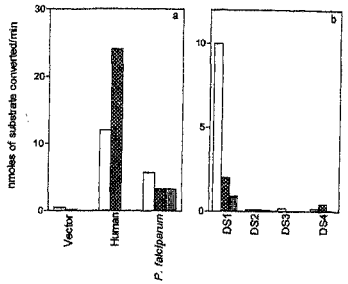

FIG. 5. Assay for HGXPRT activities in the crude *E. coli* Sϕ609 lysates transformed with wild type (a) and different chimeric (b) constructs, grown in presence of 1mM IPTG for 24 h. Amounts of conversion of hypoxanthine(▭), guanine(▩) and xanthine(▤) per minute were determined spectrophotemetrically. (See Materials and methods).

4. Discussion

HGPRT from human and *Plasmodium falciparum* exhibit variation in substrate specificity.[1, 3, 17] At the amino acid level the two sequences exhibit 78% similarity (Fig. 1). However, PfHGXPRT has high affinity for xanthine while the human does not. In this paper, the approach we have adopted to understand the role of structural features in determining substrate specificity is through construction of chimeric enzymes consisting of segments from human and PfHGPRT. The genes coding for the chimeric and wild-type HGPRTs were cloned into the expression vector pTrc99A and the *E. coli* strain Sϕ609 was transformed with the expression constructs.

Though the wild-type and chimeric HGPRTs have all been cloned into the expression vector pTrc99A, levels of expression of the different enzymes vary significantly (Fig. 3). PfHGXPRT and the chimeras DS1 and DS4, which have *P. falciparum* HGXPRT sequence at the amino terminus, yield high levels of recombinant protein in the expression system (Fig. 3, lanes 3, 4 and 7). However, human HGPRT, DS2 and DS3 (chimeras with the human HGPRT sequence at the amino terminus) do not exhibit high levels of expression upon induction with IPTG (Fig. 3, lanes 2, 5 and 6). Examination of the amino terminus of the *P. falciparum* and human HGPRT genes with respect to codon usage and ability to form stem-loop structures did not indicate either factor contributing significantly to variation in expression levels. Further experiments have to be carried out to explain the low level of expression involving modification of the codon usage at the amino terminus of human HGPRT.

pGPRT and pFPRT expressing human and *P. falciparum* HGPRTs, respectively, complement HGXPRT deficiency in Sϕ609 indicating the production of functionally active enzymes (Fig. 4). Human HGPRT phosphoribosylates hypoxanthine and guanine while the parasite enzyme has additional specificity for xanthine. Assay using crude lysates of pGPRT and pFPRT

Table II
Summary of crystal structures of HG(X)PRTs and OPRTs from different organisms

Organism	Enzyme	Residues/ subunit	Ligand	Oligomeric state in crystal	References
Human	HGPRT	217	GMP[a]	Dimer	18
T. foetus	HGXPRT	183	+/–GMP	Dimer	19
T. gondii	HGXPRT	231	Mg^{2+}, XMP[b]	Tetramer	20
E. coli	GXPRT	152		Dimer	21
E. coli	OPRT	213	SO_4^{2-}	Dimer	23
S. typhimurium	OPRT	213	PRPP, orotate	Dimer	24
S. typhimurium	OPRT	213	OMP[c]	Dimer	22

[a]GMP: Guanosine 5'-monophosphate; [b]XMP: Xanthosine 5'-monophosphate; [c]OMP: Orotate 5'-monophosphate

in Sϕ609, induced with IPTG, yielded activity profiles similar to the complementation data (Fig. 5). It should be noted that though a hyperexpressed protein band could not be seen on SDS–PAGE in the case of human HGPRT, the ability to complement HGPRT deficieny in Sϕ609 supports the production of low levels of active recombinant enzyme. Assays on the crude lysates of induced pGPRT/Sϕ609 gave high levels of activity, again indicative of expression of the recombinant human HGPRT.

Interestingly, chimera DS1, apart from retaining hypoxanthine and guanine activities, had acquired an additional specificity for xanthine. The DS1 expression clone in Sϕ609, upon induction with IPTG expresses high levels of the recombinant enzyme. The inability of DS2, DS3 and DS4 to phosphoribosylate hypoxanthine, guanine or xanthine is probably due to the inactive nature of the chimeras and is not a reflection of the low expression levels of the enzymes. Human HGPRT, though not overexpressed upon induction with IPTG, is however produced at low levels as seen from complementation analysis and enzyme assays.

Table II lists the crystal structures of HGPRTs[18–21] and OPRTs[22–24] from different organisms. The crystal structure of human HGPRT complexed with the product GMP, as deter-

Table III
Residues in human HGPRT making contacts with the guanine moiety of bound GMP

Residues	Number of contacts with guanine[a]
ILE 135	2
ASP 137	7
LYS 165	6
LYS 185	1
PHE 186	13
VAL 187	5
LEU 192	1
ASP 193	1

[a]An algorithm was written to list interatomic distances of the required cutoff in protein structures, using coordinates deposited in the Protein Data Bank. The contacts in this table were generated using a cutoff distance of 4 Å.

FIG. 6. Structure of human HGPRT[18] generated using Rasmol and the coordinates deposited in the Protein Data Bank. GMP is shown in green. The segment in DS1 replaced by *P. falciparum* HGXPRT sequence is coloured violet

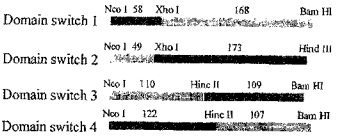

FIG. 7. Schematic representation of different chimeras showing regions from human( ) and Pf ( ) enzymes. The number of amino acids from human and *P. falciparum* HGPRT in each chimera is also indicated.

mined by Eads *et al.*[18] is shown in Fig. 6. The overall protein fold is conserved in the various HG(X)PRT structures available. The enzyme has a large barrel-shaped domain with a hood that folds over the barrel. The catalytic pocket is located in the cleft between the barrel and the hood. A flexible loop on the barrel folds over the active site during catalysis, thus protecting the oxocarbonium ion transition state from hydrolysis.[18] Residues that interact with guanine of GMP in the structure of the enzyme complex lie in the carboxy terminus of human HGPRT (Table III).

Of the four chimeric HGPRTs (Fig. 7) only DS1 was enzymatically active. In DS1, the first 58 residues correspond to PfHGXPRT sequence with the rest belonging to human HGPRT. However, unlike human HGPRT, DS1 has acquired an additional specificity for xanthine. This implies that the amino terminal domain is also involved in substrate binding, contrary to protein-base contacts seen in the crystal structure of human HGPRT complexed with GMP, which lie only in the C-terminus of the enzyme.

Examination of the crystal structures of *Salmonella typhimurium* orotate phosphoribosyltransferase (OPRT)[24] provides support for the amino terminal domain making contacts with the nitrogenous base, orotate. OPRT catalyses the phosphoribosylation of orotate to OMP and the crystal structures of the enzyme complexed with the substrates and product have been solved. The contacts OPRT makes with free orotate and with orotate in OMP are listed in Table IV. Phe34 makes 18 contacts at a cutoff distance of 4 Å with orotate in the structure of OPRT complexed with orotate and PRPP. However, this residue does not make any contact with orotate (in OMP) in the structure of OPRT complexed with the product (Table IV). Phe35 makes

Table IV
Residues in *S. typhimurium* OPRT that make contact with orotate

Residues	Number of contacts[a]	
	OPRT + OMP[b]	OPRT+orotate+PRPP[c]
LEU 25	4	8
LYS 26	3	3
PHE 34	–	18
PHE 35	4	11
THR 128	8	2
ARG 156	13	6
VAL 126	3	–

[a] The contacts in this table were generated using a cutoff distance of 4Å.
[b] Column 2 lists the protein–orotate contacts obtained from the crystal structure of OPRT complexed to the product OMP.[22]
[c] Column 3 lists the protein–orotate contacts obtained from the crystal structure of OPRT complexed with substrates orotate and PRPP.[24]

4 contacts with orotates in the structure of the enzyme complexed with OMP but this increases to 11 when complexed to substrates. There is also loss of contact in the C-terminal end of the protein when complexed to orotate and PRPP but these dramatically increase when complexed to product. This is pronounced with the C-terminal residues Thr128, Arg156 and Val126. These data indicate subtle movements of protein segments during catalysis.

In a recent report by Munagala and Wang,[25] mutation of the C-terminal, highly conserved Asp163 to Asn in *Tritrichomonas foetus* HGXPRT (TfHGXPRT) results in loss of xanthine specificity while retaining hypoxanthine–guanine activity. The alteration in substrate specificity in D163N mutant of TfHGXPRT, along with our data on DS1, indicates that multiple domains in HGPRT could be involved in determining substrate specificity. Xanthine recognition by DS1 may therefore be due to movements of the amino terminus. It is also possible that the segment switch in DS1 may be changing the active site geometry through indirect interactions. Crystal structure of DS1 will probably answer the exact nature of these interactions.

Lack of activity in DS2, DS3 and DS4 could be due to improper tertiary or quarternary structures in these chimeras. However, detailed enzymatic and kinetic analysis on the chimeras would provide a rationale for their inactivity/activity which is currently being carried out.

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