FEBS Letters 521 (2002) 72–76 FEBS 26162

A point mutation at the subunit interface of hypoxanthine–guanine–xanthine phosphoribosyltransferase impairs activity: role of oligomerization in catalysis

I.N. Sujay Subbayya, Hemalatha Balaram*

Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560 064, India

Received 5 March 2002; revised 8 May 2002; accepted 9 May 2002

First published online 22 May 2002

Edited by Judit Ovádi

Abstract Hypoxanthine–guanine–xanthine phosphoribosyltransferase (HGXPRT) from *Plasmodium falciparum* catalyzes the phosphoribosylation of hypoxanthine, guanine and xanthine. The functionally active form of HGXPRT is a tetramer but interface residues do not contribute to catalysis. Here we report the characterization of an interface mutant Y96C, which has a decreased k_{cat}, an increase in the K_m for phosphoribosyl pyrophosphate (PRPP) and no change in K_m for the purine bases when compared to the wild type enzyme. The mutant enzyme does not tetramerize in the presence of PRPP, unlike the wild type in which the tetramer is stabilized by PRPP. This is the first report of a HGXPRT mutation, at a unique interface where non-adjacent subunits interact, that impairs catalysis. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Phosphoribosyltransferase; Subunit interaction; Interface mutant; *Plasmodium falciparum*; Drug targets

1. Introduction

The purine salvage enzyme, hypoxanthine–guanine phosphoribosyltransferase (HGPRT) is an important drug target in parasitic protozoans [1]. The use of 6-mercaptopurine and 6-thioguanine in the treatment of cancer and, allopurinol in the treatment of certain parasitic diseases stems from the ability of HGPRT to convert these purine analogs into toxic metabolites [2,3]. Extensive biochemical and structural studies have been carried out on this enzyme from different organisms. In all HGPRTs studied so far, the K_m for the purine base is in the low micromolar range while the K_m for phosphoribosyl pyrophosphate (PRPP) is higher than that of the corresponding purine [4]. All HGPRTs in the crystal structures reported so far are either dimers or tetramers [4]. In solution, however, various factors such as presence of substrates, ionic strength and pH influence the oligomeric status of HGPRTs [5–8]. There is no concrete evidence for the direct participation of residues in the interfaces in catalysis as they are far away from the active site. An extensive network of hydrogen bonds links the subunits of the oligomers. Mutation of lysine68 to alanine at the dimer interface makes the human HGPRT a cooperative enzyme, indicating that cross-talk between the active sites in the subunits could be mediated by this hydrogen-bonded network [9].

The tetrameric structure of *Plasmodium falciparum* HGXPRT (hypoxanthine–guanine–xanthine phosphoribosyltransferase; HGPRT from this parasitic protozoan has an additional specificity for xanthine [10] and hence, referred to as HGXPRT in this paper) in the crystal generates three unique interfaces [11]. In this paper we represent these interfaces with respect to the loss of accessible surface area on tetramer formation, with the interface getting buried, the most being the dimer interface followed by the tetramer interface and finally, the least buried interface being called the diagonal interface. In this report we present the expression, purification and characterization of a mutant *P. falciparum* HGXPRT wherein the mutation tyrosine96 to cysteine is located in the diagonal interface. This study, for the first time, shows that a mutation in this interface of the protein quaternary structure impairs tetramer formation leading to a dramatic reduction in catalytic efficiency of HGXPRT.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used in enzyme assays including hypoxanthine, guanine, xanthine, PRPP and $MgCl_2$ were from Sigma Chemical Company, St. Louis, MO, USA. *Escherichia coli* Sϕ609 was a gift from Dr. Per Nygaard, University of Copenhagen, Denmark. Bacterial growth media were purchased from HiMedia Laboratories Limited, Mumbai, India. Hypoxanthine, guanine and xanthine stock solutions (50 mM) were made in 0.4 N NaOH. PRPP was dissolved in water. For the purpose of HGXPRT activation, PRPP, hypoxanthine and dithiothreitol (DTT) were further diluted into 10 mM potassium phosphate, pH 7.0 and immediately used.

2.2. Y96C HGXPRT construction, expression and purification

Y96C HGXPRT was obtained as a consequence of a PCR error introduced during amplification of *P. falciparum* HGXPRT gene with P2A mutation. The construction of P2A HGXPRT was reported earlier [12]. During the cloning of P2A HGXPRT in the *E. coli* expression vector pTrc99A, recombinants carrying an insert of the expected size were checked for expression of enzymatically active HGXPRT by functional complementation in *E. coli* Sϕ609 (*ara*, *Δpro-gpt-lac*, *thi*, *hpt*, *purH*, *J str A*) [13]. Colonies were grown in minimal medium using protocols described earlier [14]. One of the recombinants, which exhibited differential ability to growth in minimal medium supplemented with purine bases, was subjected to DNA sequencing and found to carry a Y96 to C mutation apart from P2 to A. Y96C HGXPRT gene was subcloned into the expression vector pET23d

*Corresponding author. Fax: (91)-80-8462766.
E-mail address: hb@jncasr.ac.in (H. Balaram).

Abbreviations: HGXPRT, hypoxanthine–guanine–xanthine phosphoribosyltransferase (EC 2.4.2.8); PRPP, phosphoribosyl pyrophosphate; DTT, dithiothreitol

PII: S0014-5793(02)02826-0

and overexpressed in the *E. coli* strain BL21(DE3). The induction conditions were the same as that reported for human HGPRT [12]. Protocols for purification of P2A and Y96C HGXPRTs were identical to that reported for wild type *P. falciparum* HGXPRT [15]. All three hyperexpressed proteins used in this study were verified for their identity on a HP 1101 electrospray mass spectrometer by direct injection of the sample. Protein estimation was done by the Bradford method using bovine serum albumin (BSA) as standard [16].

2.3. Enzyme assays

Reactions carried out at room temperature were monitored spectrophotometrically using a Shimadzu UV 1601 spectrophotometer. The conditions used for monitoring hypoxanthine, guanine and xanthine phosphoribosylation by HGXPRT were exactly as reported earlier [15]. Activation conditions were again, similar to that used for wild type HGXPRT [15]. Briefly, after purification Y96C HGXPRT was buffer exchanged into 10 mM potassium phosphate, pH 7.0. This protein was concentrated and used for activation. Activation was carried out at a protein concentration of 27 μM, in 10 mM potassium phosphate, pH 7.0, containing 200 μM PRPP, 60 μM hypoxanthine and 5 mM DTT. This mixture was stored at 4°C and activity of the enzyme was measured after 40 h of incubation. For determining the K_m of PRPP for P2A HGXPRT, PRPP concentrations were varied between 4.0 and 0.01 mM in the hypoxanthine phosphoribosylation reactions, 4.0 and 0.025 mM for the guanine and xanthine phosphoribosylation reactions. Hypoxanthine and guanine concentrations were held at 100 μM while xanthine was fixed at 300 μM for the estimation of K_m for PRPP. For determining K_m for the purines, the PRPP concentration was held constant at 1.6 mM and purine concentrations were varied between 100.0 and 5.0 μM for hypoxanthine phosphoribosylation, 100.0 and 2.5 μM for guanine phosphoribosylation and, 300.0 and 2.5 μM for xanthine phosphoribosylation. P2A concentration used in all the kinetic studies was 0.22 μM.

Hypoxanthine and guanine concentrations were maintained at 100 μM, and xanthine at 300 μM while PRPP concentrations were varied between 4.0 and 0.2 mM for estimation of K_m for PRPP for Y96C HGXPRT. PRPP was held constant at 4 mM while individual purine concentrations were varied between 100.0 and 5.0 μM for hypoxanthine phosphoribosylation, 100.0 and 2.5 μM for guanine phosphoribosylation and 300.0 and 2.5 μM for xanthine phosphoribosylation for estimating the K_ms for the purines. Y96C concentration used in all the kinetic studies was 0.54 μM. Kinetic constants for P2A and Y96C HGXPRTs were determined as reported by Subbayya et al. [15].

2.4. Size exclusion chromatography

For examining the oligomeric status of P2A and Y96C HGXPRTs, size exclusion chromatography was carried out using a Superdex-200 column (1×30 cm) attached to an Akta™ Basic HPLC. Cytochrome C (12.4 kDa), carbonic anhydrase (29.0 kDa), BSA (66.0 kDa), alcohol dehydrogenase (150.0 kDa) and β-amylase (200.0 kDa) were used as standards for gel filtration. Blue dextran was used to determine the void volume of the column. Elution was monitored at 220, 254 and 280 nm. The column was equilibrated and run with the assay buffer consisting of 100 mM Tris–HCl, pH 7.4, 12 mM $MgCl_2$ and 1 mM DTT. 100 μg protein in the same buffer was applied on to the column. Oligomeric status in the presence of PRPP was determined by equilibrating the column with 50 μM PRPP in the above buffer. 100 μg of protein preincubated for 1 h with 1 mM PRPP was used.

2.5. Structure analysis

The tetrameric structure of *P. falciparum* HGXPRT was generated by submitting the coordinates deposited in the protein data bank to European Bioinformatics Institute to generate the multimer coordinates. These coordinates were then analyzed by an in-house program to check inter-atomic distances. The program Naccess was used to determine the solvent accessibility of residues in the HGXPRT structure [17].

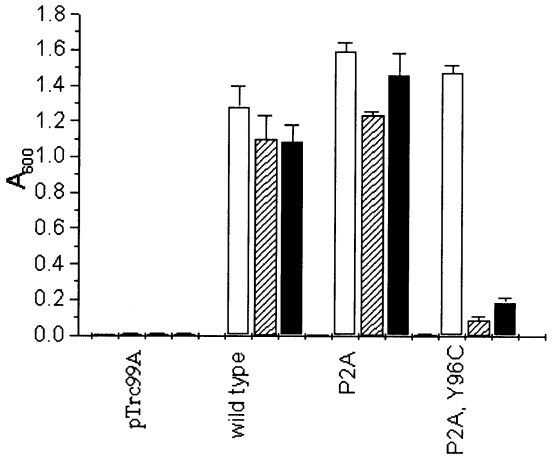

Fig. 1. Growth of *E. coli* Sϕ609 transformed with the expression constructs for wild type, P2A and Y96C HGXPRTs in minimal medium supplemented with no purine (dotted box), 500 μM hypoxanthine (□), 500 μM guanine (striped box) and 500 μM xanthine (■). Cultures were induced with 0.3 mM IPTG. A_{600} values of liquid cultures were measured after 15 h of growth at 37°C. Experiments were repeated independently at least three times and the antennae indicate the standard deviation.

3. Results and discussion

In our earlier studies on *P. falciparum* HGXPRT, the recombinant, wild type parasite enzyme was generated using the expression construct pPf1 in which the HGXPRT gene is cloned into the *E. coli* expression vector pTrc99A [14]. As a consequence of end-filling and blunt-ended ligation, the *Nco*I site was not regenerated in this construct. To facilitate easy removal of the HGXPRT gene as a cassette from expression constructs, an *Nco*I site was introduced in the 5′-end by mutation of Pro(2) (CCA) to Ala (GCA). While screening different colonies for production of enzymatically active, recombinant P2A HGXPRT by functional complementation in the HGXPRT deficient *E. coli* strain, Sϕ609, one recombinant was found to lack the ability to grow on xanthine and guanine and, could grow in minimal medium only in the presence of hypoxanthine. Sequencing of this clone revealed that apart from the introduced P2A mutation, an additional Y96C mutation (TAT to TGT) was also present. This double mutant is referred to as Y96C HGXPRT. This mutation must have been introduced by *Taq* polymerase during amplification of the P2A HGXPRT gene fragment.

Table 1
Specific activities of wild type, P2A and Y96C HGXPRTs before and after activation[a]

Enzyme	Specific activity (nmol min^{-1} mg^{-1})						Fold increase in activity		
	Unactivated			Activated					
	Hyp	Gua	Xan	Hyp	Gua	Xan	Hyp	Gua	Xan
Wild type	72	17	198	3240	2434	6490	45	141	33
P2A	49	20	207	2240	3157	9065	46	161	44
Y96C	12	7	14	87	40	229	7	6	16

[a]Protein concentrations in the assay were 0.27, 0.22 and 0.54 μM for wild type, P2A and Y96C HGXPRTs, respectively.

Table 2
Kinetic constants for the *P. falciparum* wild type, P2A HGXPRT and Y96C HGXPRT forward reactions

Enzyme	k_{cat} (s^{-1})	K_m (μM)		k_{cat}/K_m (μM^{-1} s^{-1})	
	HPRT reaction				
		Hypoxanthine	PRPP	Hypoxanthine	PRPP
Wild type	1.42	<1	27	1.42	0.05
P2A	0.89	0.7	22	1.37	0.04
Y96C	0.04	1.5	1003	0.03	3.7×10^{-5}
	GPRT reaction				
		Guanine	PRPP	Guanine	PRPP
Wild type	1.06	1.5	80	0.71	0.01
P2A	1.19	0.9	119	1.31	0.01
Y96C	0.02	3.2	4230	5.7×10^{-3}	4.2×10^{-6}
	XPRT reaction				
		Xanthine	PRPP	Xanthine	PRPP
Wild type	2.84	146	141	0.02	0.02
P2A	4.13	120	84	0.01	0.05
Y96C	0.10	189	1330	5.3×10^{-4}	7.5×10^{-5}

As shown in Fig. 1 the wild type and P2A HGXPRT were able to complement hypoxanthine, guanine and xanthine deficiencies in *E. coli* Sϕ609 with equal efficiency. Y96C HGXPRT was able to support *E. coli* Sϕ609 growth in the presence of hypoxanthine only, with guanine and xanthine phosphoribosylation activities being insignificant. These results indicate that mutation of proline2 to alanine in *P. falciparum* HGXPRT does not alter its substrate specificities and the differences seen on complementation with Y96C HGXPRT are due to mutation of tyrosine 96 to cysteine.

Though complementation studies indicated the production of active wild type, P2A and Y96C HGXPRTs from pTrc99A constructs in *E. coli* Sϕ609, large amounts of hyper-expressed protein on SDS–PAGE could be seen only in the case of wild type and P2A and, not with Y96C HGXPRT. Hence, Y96C HGXPRT was subcloned into the T7 expression vector pET23d and expression in *E. coli* BL21(DE3) yielded large amounts of recombinant protein. All the three proteins used in the study were purified using similar protocols [15], analyzed by electrospray mass spectrometry and found to have the molecular weights of 26226 (26231, wild type HGXPRT), 26204 (26205, P2A HGXPRT) and 26140 Da (26145, Y96C HGXPRT). The numbers in parentheses are the calculated molecular weights. All these masses correspond to proteins without the N-terminal methionine.

The specific activities for the wild type [15] and P2A HGXPRTs were almost identical while Y96C HGXPRT exhibited a significant drop in activity (Table 1). Surprisingly, unlike results from in vivo complementation studies in *E. coli*, xanthine and guanine phosphoribosylation activities of Y96C HGXPRT in vitro, were at levels comparable to that of hypoxanthine. IMP, the product formed from hypoxanthine is in the primary pathway of AMP and GMP formation and, hence, there is greater cellular ease in the formation of these purine nucleoside monophosphates. However, conversion of GMP to AMP involves the enzyme GMP reductase whose expression is up-regulated with increase in concentration of GMP [18]. The discrepancy between complementation and in vitro activity measurements could arise from low levels of expression of Y96C HGXPRT in Sϕ609 along with decreased specific activity for phosphoribosylation of guanine yielding concentrations of GMP, probably insufficient for the induction of significant quantities of GMP reductase required for cell growth. The high K_m for GMP (0.1 mM) for GMP reductase [19,20] could also hamper growth of cells under conditions of low in vivo concentrations of this nucleotide.

Our earlier studies with wild type *P. falciparum* HGXPRT had shown that the low levels of activity could be dramatically increased by preincubation with the substrates at high protein concentrations [15,21]. Both P2A and Y96C HGXPRTs were subjected to these activation conditions and assayed for increase in activity. Increase in activities of P2A HGXPRT was similar to that of the wild type with minor variations (Table 1) while the activity of Y96C HGXPRT, though exhibiting a 10-fold increase on activation, was at least 25-fold lower than that of activated P2A HGXPRT.

As shown in Table 2 the k_{cat}s and the K_ms for the purine bases and PRPP in the case of wild type and P2A HGXPRT show only minor variations. This again indicates that the enzymatic behavior of P2A is identical to that of the wild type, justifying the use of this mutant for all comparative studies. In the case of Y96C HGXPRT, the K_m for PRPP is highly elevated while K_m for the purine bases is not signifi-

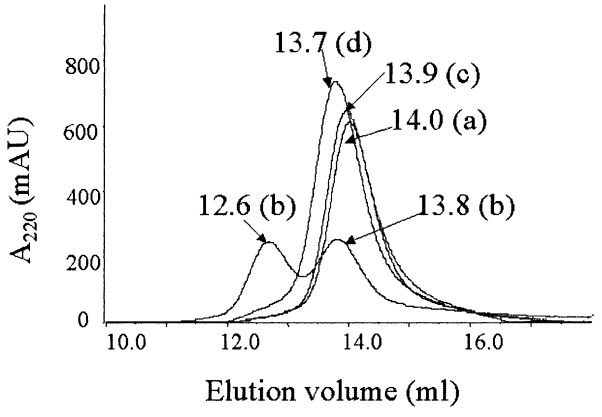

Fig. 2. Gel filtration profile of P2A and Y96C HGXPRTs. a: P2A (100 μg) in assay buffer. b: P2A (100 μg) in assay buffer with PRPP. c: Y96C (100 μg) in assay buffer. d: Y96C (100 μg) in assay buffer with PRPP. Numbers indicate elution volume (ml) of the peaks.

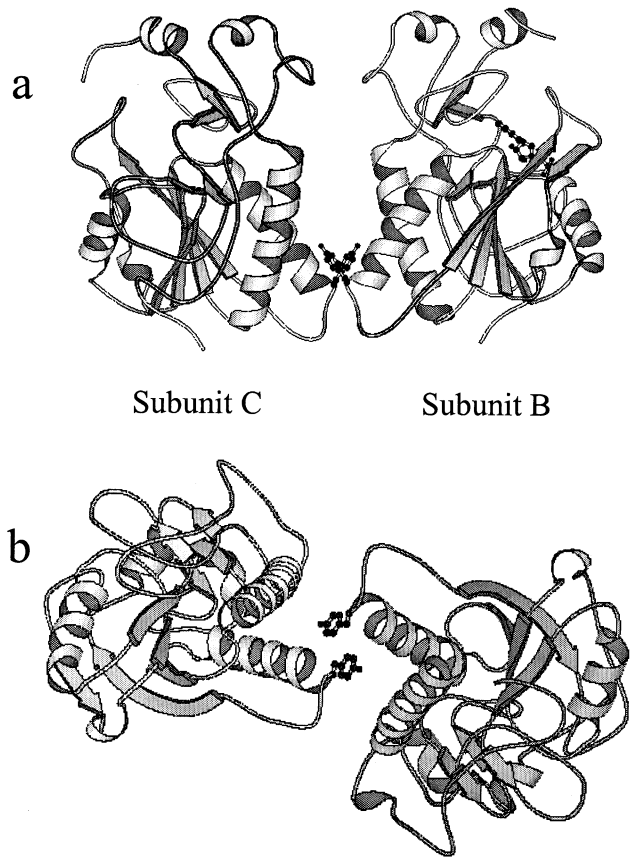

Fig. 3. a: Interaction of non-adjacent subunits B and C in *P. falciparum* HGXPRT tetramer shown along with inhibitor (ImmucillinHP) bound in the active site (Protein Data Bank, ID code 1cjb) [22]. Though all active sites are occupied in the deposited structure, this figure, for the sake of clarity, shows inhibitor complexed with subunit B only. b: Subunits B and C rotated to highlight the relative orientation of Y96 at the 'diagonal interface'. Y96 is shown in ball and stick in (a) and (b). These figures were generated using Molscript [28].

cantly altered (Table 2). Hence, in the kinetic studies of Y96C HGXPRT, PRPP concentrations were maintained at 4 mM to determine K_m for the purine bases. However, the reaction velocity of this mutant did not saturate even at this high concentration of PRPP. Hence, the PRPP K_ms for Y96C, reported in this paper, might be lower than the actual values, with purine base K_ms being apparently higher.

Our earlier studies have shown that the oligomer formation of *P. falciparum* HGXPRT is dependent on ionic strength of the buffer and presence of PRPP. The enzyme is a dimer in the assay buffer (100 mM Tris–HCl, pH 7.4, 12 mM $MgCl_2$) with addition of PRPP facilitating tetramer formation. These studies on the wild type enzyme have also shown that activation from a low-activity form to a high-active form involves conversion from dimers to tetramers (unpublished observations). Fig. 2, that summarizes the results obtained from size exclusion chromatography analysis of P2A and Y96C HGXPRTs, indicates that in the former enzyme like with the wild type, tetramer formation is seen on addition of PRPP. Like the wild type, increase in activity is seen with P2A on activation with PRPP and hypoxanthine. However, Y96C HGXPRT remains a dimer even on incubation with PRPP (Fig. 2). Activity of Y96C HGXPRT does increase many fold on activation (Table 1), indicating that low levels of active tetramers are indeed being formed. These tetramers are probably not as stable as in the wild type and P2A HGXPRT, consequently the specific activities never reach a high level.

Examination of the tetrameric HGXPRT structure indicates that Y96 is located in an interface, which involves cross-talk between non-adjacent subunits (Fig. 3a,b). Analysis of solvent accessibility of Y96 indicates that this residue in the monomer is 22% buried. On dimerization the buried surface of this residue is 26%, with tetramerization increasing buried area to 76%. Formation of HGXPRT dimer, leads to 2812 Å^2 of the total surface area getting buried, which increases by further 2070 Å^2 on tetramerization [11]. Of this total area of 4882 Å^2, which gets buried, 135 Å^2 is contributed by Y96. The disposition of the various residues surrounding Y96 in the tetrameric HGXPRT, at a distance of 4 Å is shown in Fig. 4. The diagonal interface contacts arise from the interactions of Y96 in subunit B with E55, R92, I93 and Y96 in subunit C. Similar contacts are seen between subunits A and D.

The studies on Y96C reported in this paper indicate that replacement of tyrosine96 by cysteine, which leads to a reduction in occupied surface area by 90 Å^2, results in dramatic impairment of HGXPRT activity. The introduction of a smaller residue, cysteine, may create a cavity at this interface making the tetramer unstable. Formation of tetrameric HGXPRT on addition of PRPP and the need for activation hint at subtle rearrangements being induced in individual subunits on oligomerization (unpublished observations). The exact nature of these rearrangements is not clear. However, structural evidence exists for conformational changes that occur in individual subunits on substrate binding [23]. The predominant motion is that of the active site loop II that flips by 25 Å to cover the active site when ligands are bound [24]. In a recent study, it has been found that inhibitor binding stabilizes a unique segment located at the 'diagonal interface' of the active tetramer in human HGPRT. In the human enzyme, the residue corresponding to Y96 is N87 and it has been shown by H/D exchange that the region around this residue in the protein exchanges hydrogen at a slower rate on inhibitor binding [6].

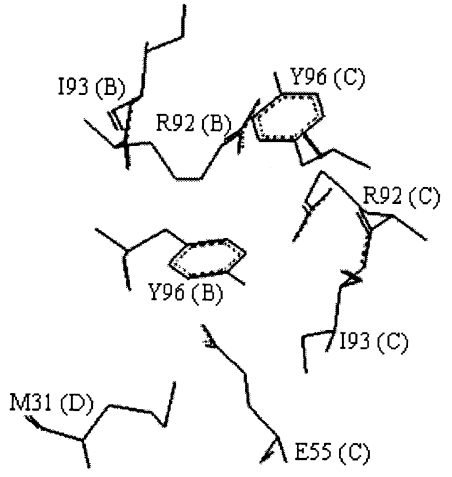

Fig. 4. Residues contacting Y96 in subunit B at a distance of 4 Å in the HGXPRT tetramer. Letters in parentheses indicate the subunit. Two adjacent residues on either side of Y96 were ignored for computing the contacts.

In HGXPRT, Y96 is located at the C-terminal end of helix3 and is more than 10 Å away from the catalytic pocket in any of the four subunits of the tetramer (Fig. 3a). Sequence comparison of this segment with other HGPRTs indicates the presence of large diversity in this segment. Small molecules designed to disrupt the tetramer could therefore, be targeted to this unique region in HGPRTs. Non-active site segments crucial for oligomerization, but which are unique to different species, can therefore be exploited in species-specific drug design. Such an approach has been successfully exploited in the case of triose phosphate isomerase from *P. falciparum*, thymidylate synthase from *Lactobacillus casei* and the HIV protease [25–27].

Acknowledgements: We thank Dr. Per Nygaard for providing the *E. coli* strain Sϕ609. Mass spectra were recorded at the Department of Biotechnology funded facility at the Indian Institute of Science, Bangalore. We acknowledge the DNA sequencing facility, Indian Institute of Science, Bangalore for sequencing all the constructs used in this report. This work was supported by grants from the Council for Scientific and Industrial Research, Department of Biotechnology, Department of Science and Technology, Government of India, and Science Institutes Group Innovative Research Grant, Princeton, USA. I.N.S.S. was supported by Predoctoral fellowship by the Council for Scientific and Industrial Research.

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