

Synthetic peptides as inactivators of multimeric enzymes: inhibition of *Plasmodium falciparum* triosephosphate isomerase by interface peptides

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Abstract Synthetic peptides corresponding to two distinct segments of the subunit interface of the homodimeric enzyme triosephosphate isomerase (residues 9–18, ANWKNGTLE, peptide I; residues 68–79, KFGNGSYTGEVS, peptide II) from *Plasmodium falciparum* (PfTIM) have been investigated for their ability to act as inhibitors by interfering with the quaternary structure of the enzyme. An analog of peptide II containing cysteine at the site corresponding to position 74 and tyrosine at position 69 in the protein sequence KYGNGSCTGEVS (peptide III) was also investigated. A substantial fall in enzyme activity was observed following incubation of the enzyme with peptide II, whereas peptide I did not show any appreciable inhibition. The inhibitory effect was more pronounced on two mutants of PfTIM (Y74C and Y74G), both of which have reduced stability compared to the wild-type protein due to an interface cavity. The IC₅₀ value determined for peptide II is in the range of 0.6–0.8 μM. This study suggests that interface peptides of oligomeric enzymes can be used to inhibit dimeric enzymes by disrupting their native multimeric states and may provide lead structures for potential inhibitor design. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Malaria; Interface peptide; Enzyme inhibition; *Plasmodium falciparum* triosephosphate isomerase

1. Introduction

Inhibition of metabolic enzymes is an important approach in the development of new therapeutics. In the case of parasitic diseases, selective inhibition of pathogen enzymes is a desirable goal. In malaria, the intraerythrocytic stage of the parasite, *Plasmodium falciparum*, is characterized by a marked increase in glycolytic flux, as this is the major source for metabolic energy for the parasite [1]. Glycolytic enzymes from *P. falciparum* are being structurally characterized in the hope that they may serve as potential targets for inhibition. Thus far, structures have been reported for triosephosphate isomerase (TIM), lactate dehydrogenase and aldolase [2–4]. The multimeric nature of many of these enzymes permits consideration of two distinct approaches towards inhib-

itor design: (i) inhibitors that target the active site and (ii) molecules that disrupt subunit–subunit interfaces and interfere with protein assembly. The former approach is limited by the high structural conservation of enzyme active sites, which often precludes an effective distinction between the host and the pathogen enzymes. The greater structural variability of protein–protein interfaces suggests that subunit contact sites may provide a possible target for the efficient differentiation between the host and parasite enzymes [5]. The use of dimer interface peptides as inhibitors has been explored in several cases [6]. Some representative examples are *Lactobacillus casei* thymidylate synthase [7], HIV-1 protease [8–11], restriction endonuclease *EcoRI* [12], herpesvirus ribonucleotide reductase [13,14] and human glutathione reductase [15].

Among the glycolytic enzymes, TIM is a particularly attractive test system for exploring inhibition strategies, as a vast body of literature is available on its structure and biochemistry [16–23]. TIM is a prototype of the β₈/α₈ TIM barrel fold, and the active site of the enzyme consists of Lys 12, His 95 and Glu 165. It catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [24]. Crystal structures from 10 different sources are available for TIM, whereas complete sequence information is available from as many as 144 different organisms, enabling detailed structural and sequence comparisons. TIM is a homodimeric protein except in *Thermotoga maritima* and *Pyrococcus woesei*, where it is tetrameric [25,26].

In this report we describe the effect of synthetic peptides derived from the subunit interface of *P. falciparum* TIM (PfTIM) on enzyme activity. It was found that among the peptides used, peptide II, which corresponds to loop 3 of the protein, shows much greater inhibition compared to peptide I (loop 1). The inhibition was much more pronounced in the case of the two mutant proteins of PfTIM, Y74C and Y74G, in which the introduction of cavities destabilizes the subunit interface.

2. Materials and methods

2.1. Chemicals

α-Glycerol phosphate dehydrogenase, NADH and glyceraldehyde-3-phosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, MO, USA, and used without further purification. The substrate glyceraldehyde 3-phosphate was obtained as a diethyl-acetal monobarium salt and processed to its active form according to the manufacturer's instructions. The concentration of glyceraldehyde 3-phosphate extracted was estimated using glyceraldehyde-3-phosphate dehydrogenase. Chemicals used in solid phase synthetic proce-

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Abbreviations: PfTIM, *Plasmodium falciparum* triosephosphate isomerase (EC 5.3.1.1)

dures were obtained from Novabiochem (Nottingham, UK), Bachem (Bubendorf, Switzerland) or Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and piperidine were purchased from Chem-Impex International Inc. (Wood Dale, IL, USA). The resin (PAL-PEG-PS) used in peptide synthesis was purchased from PerSeptive Biosystems (Hertford, UK). All other chemicals were procured locally.

2.2. Purification of PftIM and its mutants

The PftIM gene was cloned into a pTrc99A vector called pARC1008 [27]. The enzyme was overexpressed in the *Escherichia coli* strain AA200, which has a null mutation in its host TIM gene [28]. Site-directed mutants Y74C and Y74G were constructed as reported earlier [17]. The overexpressed protein was purified by a standardized protocol [2,17]. The purified preparations were checked on electrospray ionization mass spectrometry (ESI-MS). Specific activities of the three proteins wild-type TIM, Y74C and Y74G are 7800, 4800 and 400 U/mg, respectively.

2.3. Peptide synthesis

The interface peptides were synthesized on a LKB-Biolynx 4175 semi-automatic peptide synthesizer using standard Fmoc (9-fluorenylmethyloxycarbonyl) chemistry. The peptides were acetylated at the N-terminus, prior to cleavage using 1:1 acetic acid/acetic anhydride. The resin-assembled peptides were cleaved by treating with 95% TFA for 4 h, in the presence of anisole (2.5%) and ethanedithiol (2.5%), and purified by reverse phase high performance liquid chromatography using a C18 column (5–10 μm , 7.8×250 mm). The pure peptides were characterized by ESI-MS using a Hewlett Packard 1100 LC MSD mass spectrometer; peptide I: $M_{\text{obs.}} = 1136.1$ Da ($M_{\text{cal.}} = 1135.3$ Da); peptide II: $M_{\text{obs.}} = 1288.8$ Da ($M_{\text{cal.}} = 1287.3$ Da); peptide III: $M_{\text{obs.}} = 1244$ Da ($M_{\text{cal.}} = 1243.3$ Da). Concentrations of the peptides were determined by either tyrosine ($\epsilon_{275} \approx 1420 \text{ M}^{-1} \text{ cm}^{-1}$) or tryptophan absorbance ($\epsilon_{280} \approx 5600 \text{ M}^{-1} \text{ cm}^{-1}$) [29].

2.4. Enzyme assay

Kinetic measurements were carried out according to the method of Plaut and Knowles [30] on a Shimadzu UV210A double beam spectrophotometer at room temperature. The cuvette contained 100 mM triethanolamine buffer, pH 7.6, 5 mM EDTA, 0.5 mM NADH and α -glycerol phosphate dehydrogenase (20 $\mu\text{g/ml}$) and 0.16–2.4 mM glyceraldehyde 3-phosphate. Enzyme activity was determined by monitoring the decrease in absorbance at 340 nm. The dependence of the initial rate on the substrate concentration was analyzed according to the Michaelis–Menten equation. The values for the kinetic parameters (K_m and k_{cat}) were calculated from Lineweaver–Burk plots.

2.5. Peptide inhibition

200 nM of PftIM wild-type enzyme was preincubated at three different concentrations of each of the three peptides in ratios 1:10 (2 μM peptide), 1:100 (20 μM peptide) and 1:1000 (200 μM peptide) for 1 h. 1 μl of this solution was then diluted to 1 ml of the final reaction mixture in the cuvette to get a final enzyme concentration of 0.2 nM (5 ng/ml). Similarly, the inhibitory effect of the three peptides was tested for the two mutants Y74C and Y74G using concentrations of 0.2 μM and 2 μM per reaction, respectively. The control sample had the enzyme in the same concentration. The activities were compared with and without peptide. Each set was done in triplicate and the data were averaged.

3. Results

3.1. Design of interface peptides

The peptides were designed after detailed structural analysis of the crystal structure of PftIM that was solved to 2.2 \AA [2]. The interface is largely comprised of loops with loop 1 (residues 10–16) and loop 3 (residues: 66–79) involved in extensive intersubunit interactions (Fig. 1) [2]. The residue composition at the interface is largely polar. Fifteen amino acids have uncharged polar side chains including three glycine residues, seven amino acids have charged polar side chains and only eight amino acids are non-polar. The delineation of residues which constitute the subunit interfaces of PftIM was carried

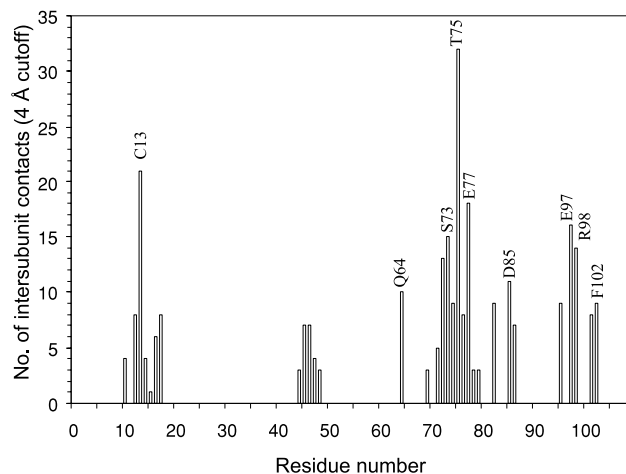


Fig. 1. A stick plot showing residue contacts across the subunit interface in PftIM structure (PDB code: 1ydv). Distance cutoff was 4.0 \AA . Only residues 1–105 are shown, as residues beyond 102 do not make any contacts at the dimer interface.

out using two approaches: (i) a listing of all interresidue contacts between groups on the interacting subunits using an interatomic cutoff distance of 4 \AA . Fig. 1 shows an intersubunit contact plot, which clearly demarcates the residues at the interface. It is seen that the interface consists of discontinuous peptide segments, of residues 10–17, 44–49, 69–79 and 95–102. Two of these regions, loop 1 and loop 3, span more than five consecutive residues along the polypeptide chain and were chosen for inhibition studies. (ii) Interface residues were also identified using differential surface accessibility values calculated for residues in both dimeric and monomeric structures [31]. This approach yielded identification of residues almost identical to that obtained in Fig. 1.

Peptide I corresponds to loop 1 of the protein, residues 9–18, ANWKCNGTLE. Loop 1 connects the first β -strand to the first α -helix. This region encompasses one of the active site residues, Lys 12. This stretch is highly conserved among all known TIM sequences. Interestingly, loop 1 also contains Cys 13, which has been earlier reported as a potential site for drug targeting in the case of trypanosomal and leishmanial TIMs, as the corresponding residue in human TIM is methionine [32–35]. In *Plasmodium*, Cys 13 is involved in extensive interactions across the interface with atoms of Asn 71, Ser 79 and Glu 77 of the other subunit.

Peptide II corresponds to loop 3 of the protein, residues 68–79, KFGNGSYTGEVS. Loop 3 protrudes ~ 13 \AA out of the bulk of the monomer and docks into a narrow pocket close to the active site of the other subunit [2]. It contributes substantially to intersubunit interactions and is very important for dimer stability, as $\sim 80\%$ of the intersubunit atom–atom contacts involve this loop, a feature noted earlier for other TIMs [17,36–38]. Tyr 74 makes several critical contacts at the dimer interface, particularly with residues Cys 13, Glu 97 and Tyr 101 from the other subunit, and is also a part of an aromatic cluster involving residues Phe 69 from the same subunit and Tyr 101 and Phe 102 from the other subunit. Aromatic clusters have frequently been implicated in the stabilization of folded protein structures. Quadrupole–quadrupole interactions involving proximal aromatic rings have been suggested to be important contributors to protein stability [39,40]. It was earlier observed that on mutating tyrosine to cysteine

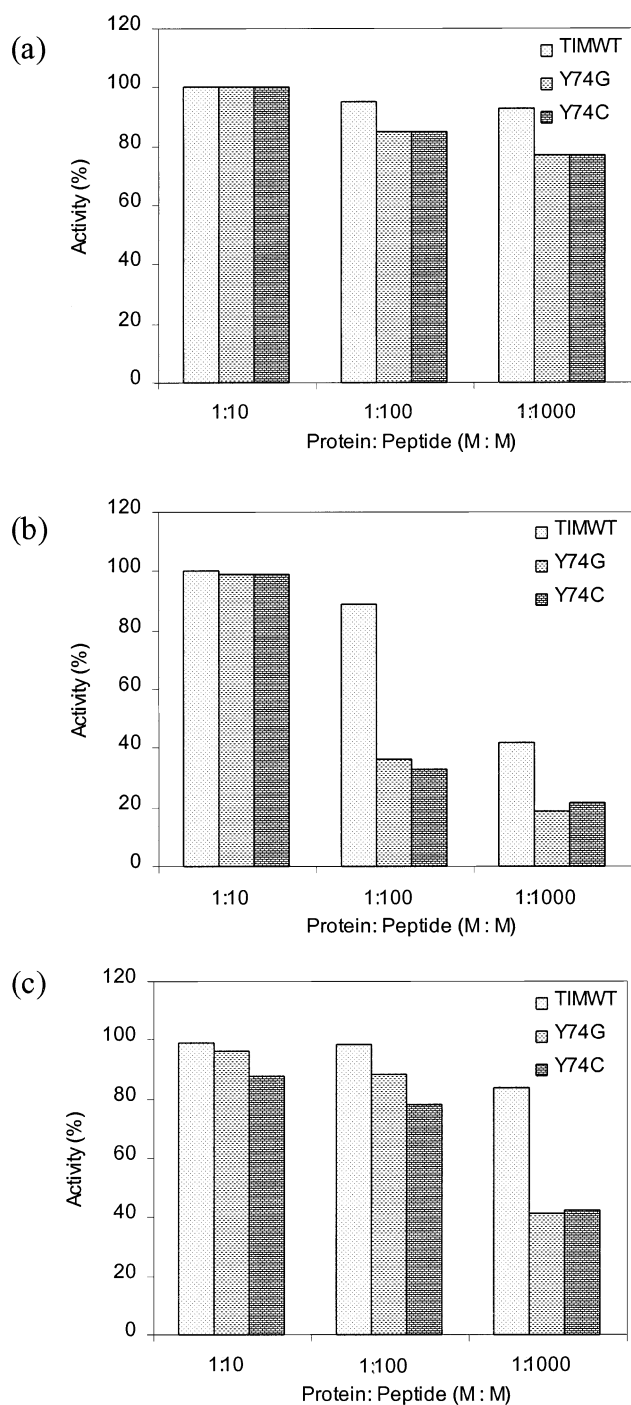


Fig. 2. Bar diagram showing the percentage fall in the enzymatic activity of the wild-type and the mutant enzymes on incubation with the peptide. 100% for each represents the maximum specific activity observed in absence of the peptide. a: Peptide I. b: Peptide II. c: Peptide III.

(Y74C) a cavity at the interface was created, which leads to weakening of subunit–subunit interactions. Prolonged aerial oxidation of this cysteine gave rise to a covalent cross-link with Cys 13 of the other subunit. This oxidized form of the mutant, which contains two symmetry-related intersubunit disulfide bonds, was found to be appreciably stabilized, relative to the reduced form [17,18]. The Y74G mutant was significantly destabilized due to the interface cavity and showed appreciable dissociation of the dimer at low protein concentrations, with a concurrent loss of enzymatic activity (unpublished data). This suggests that the interface interactions are quite essential for the stability of the enzymatically active dimer.

Peptide III (KYGNNGSCTGEVS) is an analog of peptide II with the replacements Y74C and F69Y (note that for convenience we adopt the protein residue numbering scheme in the peptide fragments). In principle, the presence of Cys 74 should permit covalent cross-linking of the peptide, in a manner similar to that used earlier in the case of HIV-1 protease, where an inhibitor was designed to form a disulfide bond with Cys 95 present at the dimerization interface of HIV-1 protease [41].

3.2. Inhibition studies

Inhibition of enzymatic activity of wild-type PftIM and two of its mutants, Y74C and Y74G, by these peptides was studied. Earlier, we have shown that both mutants have much weaker dimeric interactions as compared to the wild-type protein due to the incorporation of a cavity at the interface [17]. Table 1 summarizes the kinetic properties of these three proteins. It can be seen clearly that the interface mutation destabilizes the subunit interactions and leads to a fall in the enzymatic activity, which is more pronounced in the Y74G mutant.

The effect of interface peptides on enzymatic activity of the wild-type and the mutant enzymes is shown in Fig. 2. Peptide I showed only negligible inhibition of wild-type, Y74G and Y74C enzyme activities with a fall in the enzymatic activity by only ~20% at 1000-fold molar excess of the peptide. Peptide II was found to be an efficient inhibitor with the activity falling to ~45% at 1000-fold molar excess of the peptide in case of the wild-type enzyme. In the case of either of the mutants, Y74C or Y74G, even at 100-fold molar excess of the peptide, only ~30% activity could be obtained. Peptide III was also found to inhibit the activity of the mutant enzymes with about ~40% activity remaining in the presence of 1000-fold molar excess of the peptide (concentrations used were similar to those used for peptide II). However, it was found to be much less effective against the wild-type enzyme, where more than ~80% of the activity could be obtained, at a similar concentration of the peptide. The kinetics of inhibition were not investigated in detail. However, there was no further increase in inhibitory effect after 1 h and the observed fall in activity was similar even after 24 h of incubation (data not shown), suggesting that optimal inhibition was achieved with-

Table 1
Kinetic properties of wild-type TIM and its mutants

Property	Wild-type TIM	Y74C	Y74G
Specific activity (U/mg)	7800–8000	4500–5000	360–430
K_m (mM)	0.35 ± 0.16	0.33 ± 0.15	0.34 ± 0.076
k_{cat} (min^{-1})	$2.68 \pm 0.84 \times 10^5$	$1.45 \pm 0.34 \times 10^5$	$0.071 \pm 0.016 \times 10^5$

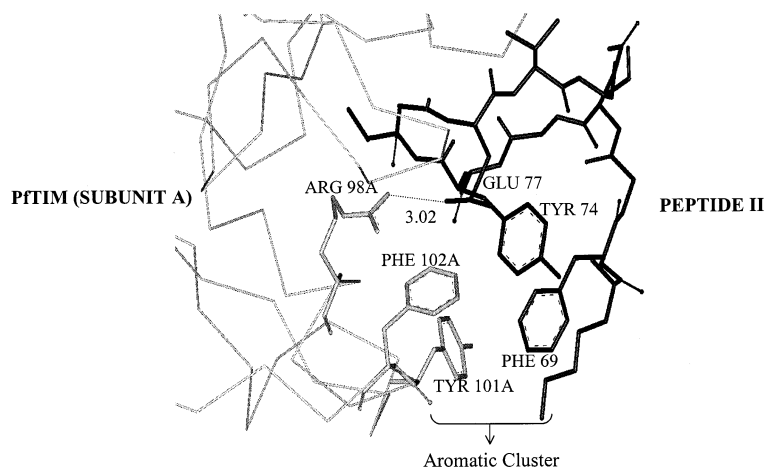


Fig. 3. Three-dimensional view of the possible interactions between subunit A of PfTIM (gray) and peptide II (black). A salt bridge between Arg 98 and Glu 77 and aromatic–aromatic interactions between Tyr 101, Phe 102 and Phe 69 and Tyr 74 are shown.

in relatively short incubation times. Indeed, extremely rapid inhibition was obtained in a study of *Trypanosoma brucei* TIM by cyclic hexapeptides [43].

4. Discussion

The observed fall in the enzymatic activity to as low as 45% at 1000-fold molar excess of peptide II ($\sim IC_{50} = 0.6\text{--}0.8 \mu\text{M}$), in the case of inhibition of wild-type enzyme, suggests that this segment of the interface may in fact contribute substantially to the dimer stability. The importance of an aromatic residue at position 74 is emphasized by the reduced inhibition observed in case of peptide III, in which Tyr 74 is replaced by Cys. The enhanced inhibition observed with PfTIM mutants harboring destabilizing interface mutations provides support for the view that peptide inhibition is indeed mediated by interaction with interface segments. The TIM interface is largely composed of irregular loop structures. All three interface peptides showed the absence of any appreciable secondary structure in aqueous medium, yielding a single negative CD band at 200 nm. Furthermore, the three synthetic segments also did not show any induction of secondary structure in structure-inducing solvents like 2,2,2-trifluoroethanol and hexafluoroacetone (data not shown). Inspection of the PfTIM crystal structure reveals that the segment corresponding to peptide II (Fig. 3) adopts a folded conformation which brings the aromatic rings of Phe 69 and Tyr 74 into proximity. This pair further interacts with an aromatic pair from the protein, Tyr 101 and Phe 102 [2]. A conformation of the loop segment corresponding to peptide II contains a type II β -turn centered at Thr 75 and Gly 76. Stabilization of this reverse turn feature in further analogs merits investigation. Interestingly, sequence-unrelated cyclic hexapeptides have been shown to be effective inhibitors of trypanosomal TIM [42–44]. The present study demonstrates that the use of dimer interface peptides is a viable strategy in generating lead sequences for inhibitor design.

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