Plasmodium falciparum triosephosphate isomerase: New insights into an old enzyme*

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Abstract: Triosephosphate isomerase (TIM), a central enzyme in the glycolytic pathway, has been the subject of extensive structural and mechanistic investigations over the past 30 years. The TIM barrel is the prototype of the $(\beta/\alpha)_8$ barrel fold, which is one of the most extensively used structural motifs in enzymes. Mechanistic studies on TIM from a variety of sources have emphasized the importance of loop 6 dynamics for enzyme activity. Several conserved residues in TIM have been investigated by extensive site-directed mutagenesis of the enzyme from yeast, chicken, and trypanosoma. The cloning and sequencing of the TIM gene from the malarial parasite *Plasmodium falciparum* in 1993 revealed the unexpected mutation of a hitherto conserved residue serine (S96) to phenylalanine (F96). Subsequent results from the genome sequencing programs of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium yoelii* confirmed the presence of the S96F mutation in malarial parasites. The crystal structure of PfTIM and several inhibitor complexes, including a high-resolution (1.1 Å) structure of the PfTIM 2-phosphoglycerate complex, revealed that loop 6 had a propensity to remain open, even in several ligand bound structures. Furthermore, both open and closed forms could be characterized for the same complex. Since glycolysis is the primary source of ATP for the malarial parasite during the intraerythrocytic stage, glycolytic enzymes present themselves as potential targets for inhibitors. Two distinct approaches have been explored. The use of dimer interface peptides, which interfere with assembly, has proved promising. Inactivation of the enzyme by modification of a cysteine (C13) residue, which lies close to the active site residue, lysine (K12) is another potential strategy. The differential reactivity, of the four-cysteine residues, at positions 13, 126, 196, and 217 in each subunit has been established using electrospray ionization mass spectrometry. Studies of single tryptophan mutants (W11F and W168F) of PfTIM provide a probe to study folding, stability, and inhibitor interactions.

INTRODUCTION

Triosephosphate isomerase (TIM) is a well-studied, glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Banner et al. [1] reported the first crystal structure of TIM from chicken breast muscle in 1975. The structure has eight α-helices alternating with eight β-strands along the polypeptide chain. The parallel β-strands are hydrogen-bonded to each other and form a central, solvent-excluded, eight-stranded β-barrel. The eight helices wrap around the central β-barrel. The strands and helices are arranged approximately antiparallel to each other. This

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fold is popularly known as the "TIM barrel" fold, as it was the first structure to be determined in this family. A large body of structure–function correlations based on site-directed mutagenesis of the enzymes from yeast, chicken, rabbit, and trypanosoma have provided many insights into catalytic mechanisms [2–5]. Knowles states that "this enzyme (TIM) appears to have arrived at the end of its evolutionary development as a catalyst" [6]. Specific roles have been ascribed to many of the conserved residues like glutamate, histidine, and lysine. Cloning and sequencing of the TIM gene from the malarial parasite *Plasmodium falciparum* revealed interesting differences from those of other reported TIMs, notably serine 96(S96); a completely conserved residue in all TIM sequences hitherto reported was replaced by a phenylalanine (F) residue [7]. The availability of the recombinant parasite enzyme prompted us to undertake detailed analysis of the effects of site-directed mutations on folding, stability, and activity [8–11].

The search for new antimalarial drugs continues with unabated vigor because of the increased spread of the disease, with *P. falciparum* infection causing the most lethal form of malaria. The growing problem of drug resistance in malaria highlights the need to quickly develop effective, new antiparasitic agents. In the intraerythrocytic phase of the parasite life cycle, glycolysis is estimated to be the predominant pathway for ATP production, making glycolytic enzymes an attractive target for inhibitory intervention [12–13]. Malarial parasites appear to lack a functional TCA cycle. Three-dimensional structures are available for all the enzymes of the glycolytic pathway, with at least one member of each class structurally characterized from different sources. However, there is limited amount of structural information on glycolytic enzymes from *P. falciparum*, as shown schematically in Fig. 1.
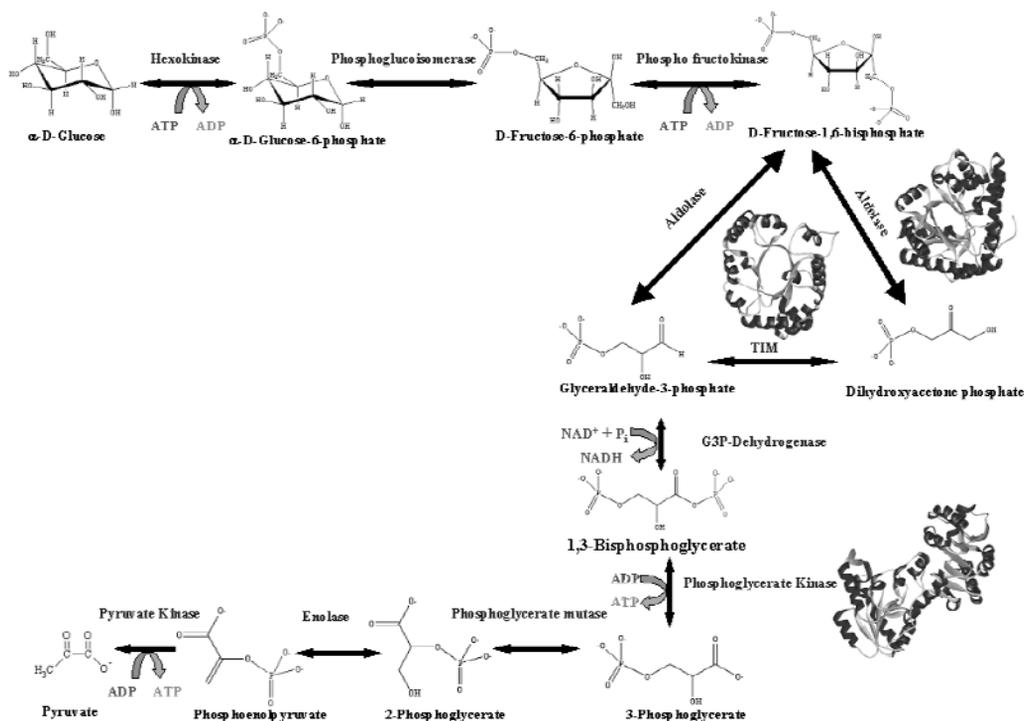

Fig. 1 Glycolytic pathway, with available crystal structures of proteins from *Plasmodium* species. Only monomeric structures of the enzymes are shown; TIM [14], aldolase [15], and phosphoglycerate kinase [16] are illustrated using PDB coordinate sets 1YDV, 1A5C, and 1LTK, respectively.

Triosephosphate isomerase from *P. falciparum* (PfTIM) was cloned and expressed by Ranie et al. in *E. coli* [7]. Table 1 compares the kinetic properties of PfTIM with the enzymes from other sources. Figure 2 shows the sequence alignment of TIM from various organisms. In addition to the S96F mutation, another important difference between the human and Plasmodium TIMs is the M13C mutation, which occurs at the dimer interface. The S96F mutation is also observed in other Plasmodium species such as *Plasmodium vivax* and *Plasmodium yoelii*. The M13C mutation is also seen in other parasitic TIMs (e.g., *Trypanasoma*, *Leishmania*). In principle, differences between human and parasite enzymes are of interest in the design of selective inhibitors. PfTIM has been shown to be an extremely robust $(\beta/\alpha)_8$ barrel which retains considerable structure even in 8 M urea [8]. Biophysical studies on folding and stability of the wild-type enzyme and on interface mutants have permitted an analysis of the unfolding process. The interface mutants have also permitted the dissection of the processes of subunit dissociation and polypeptide unfolding [9–10].

Table 1 Kinetic properties of TIM from different sources for the conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate [10].

Source	K_m (mM)	$k_{cat} \times 10^5$ (min^{-1})
P. falciparum	0.35 ± 0.16	2.68 ± 0.84
Chicken	0.47	2.56
Yeast	0.62 ± 0.05	1.41 ± 0.36
T. brucei	0.25 ± 0.05	3.7
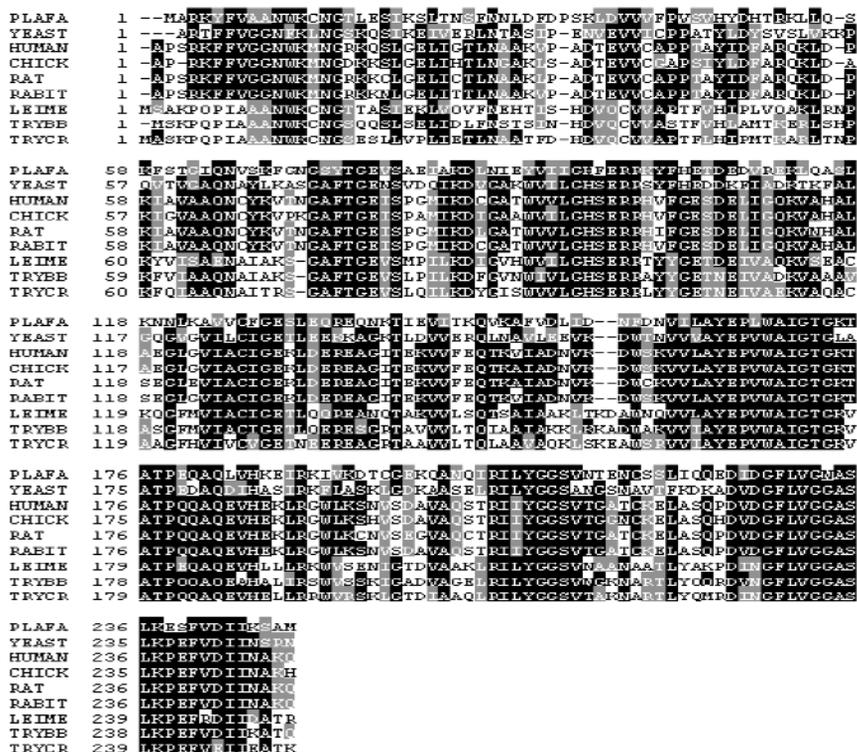
Fig. 2 Sequence alignment of triosephosphate isomerase from different sources. The alignment was performed using the Clustal W (multiple alignment) program.

STRUCTURES OF PfTIM INHIBITOR COMPLEXES AND LOOP 6 DYNAMICS

The crystal structure of PfTIM was first reported at 2.2 Å, in 1997 [14]. Figure 3a shows a view of the fold of the monomer illustrating the active site residues (H95, K12, E165). Figure 3b is a view of the dimer interface and also shows the location of Trp11, Trp168, and Cys-13. Strategies for enzyme inhibition generally target active sites or the subunit interfaces in the case of multimeric enzymes [17–18].
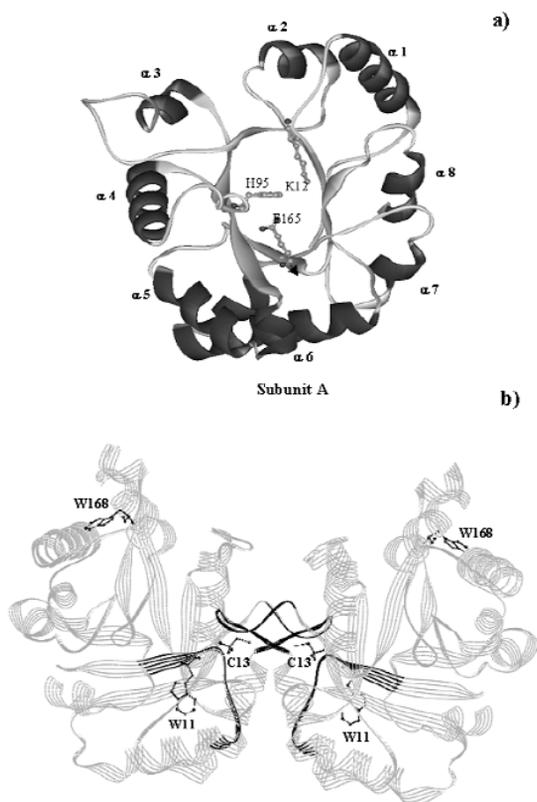
Fig. 3 Structure of PfTIM. (a) monomer (subunit A) with active site residues shown. (b) PfTIM dimer with residues W11, W168, and C13 marked. The dark region in the figure shows the dimer interface.

The structures of several PfTIM inhibitor complexes have been determined (Table 2) [19–21]. The high-resolution structure of the PfTIM-2-phosphoglycerate complex has been reported at 1.1 Å [21]. A noticeable feature of the PfTIM inhibitor complexes is the tendency of loop 6 (residues from 166–176) to remain in the *open* conformation, even when the active site is occupied by a ligand. This is in contrast to the structures of TIMs from other sources, where loop *open* forms are observed for un-ligated enzymes and loop *closed* forms are observed upon ligand binding. The sole exception is the structure of the trypanosomal TIM complex with *N*-hydroxy-4-phosphonobutanamide, where a loop *open* form was previously characterized [22]. Figure 4 shows a comparison of the loop *open* and *closed* states of PfTIM, illustrating a movement of approximately 6–7 Å of the tip of the loop upon closure. A possible reason advanced for the tendency of loop 6 to remain open in the ligand bound state in PfTIM was the potential steric clash between F96 and I170 in the *closed* form [19]. The crystal structure of the PfTIM-phosphoglycolate complex yielded two crystal forms orthorhombic ($P2_12_12_1$) and monoclinic (*C*2). Curiously, in the orthorhombic form loop 6 was in an *open* conformation, while in the monoclinic form the *closed* conformation was observed. In the latter form, steric clashes were avoided by F96 adopting an alternate side-chain orientation. In the 1.1 Å structure of the PfTIM 2-phosphoglycerate

complex, the protein dimer forms the asymmetric unit, with one subunit showing ligand occupancy with the *loop* closed, while the other has loop 6 in an *open* conformation.

**Table 2** Crystal structures of PfTIM–ligand complexes.

PDB code (ref.)	Resolution (Å)	Ligand	Space group	Loop status
1YDV (14)	2.2	–	C_12_1	Open
1LYX (19)	1.9	Phosphoglycolate	$C2$	Closed
1LZO (19)	2.8		$P2_12_12_1$	Open
1M7P (20)	2.4	Glycerol-3-phosphate	$P2_1$	Open
1O5X (21)	1.1	2-Phosphoglycerate	$P2_1$	Open
1M7O (20)	2.4	3-Phosphoglycerate	$P2_1$	Open

Fig. 4 Superposition of structures of PfTIM in loop *open* and *closed* states with the inhibitor phosphoglycolate [19].

Loop closure upon substrate binding has been suggested to be important in preventing the formation of the cytotoxic byproduct, methyl glyoxal. Loop 6 moves as a rigid body about hinge residues (*N*-terminal hinge 166–167 and *C*-terminal hinge 174–176). Solid-state NMR studies have shown that the loop movement is an inherent property of the enzyme and the nature of the bound ligand affects only the relative populations of the *open* and *closed* conformations [23]. Recent solution NMR studies have also provided evidence for the view that loop motion and product release are concerted and might be one of the rate-limiting steps during catalysis [24]. NMR studies have also given an estimate of the rate of loop movement in TIM, on the order of 10^4 s^{-1} [25].

The presence of a tryptophan residue (W168) on loop 6 suggests the possibility of using fluorescence spectroscopy to study loop motion and inhibitor binding. PfTIM has only two tryptophan residues—W11 and W168. We have, therefore, constructed the single Trp mutants W11F and W168F, permitting the deconvolution of the fluorescence spectrum of the wild-type enzyme into contributions from individual Trp residues. Figures 5a and 5b demonstrate the significant differences in the emission maximum and quantum yields for the two Trp residues [26]. Notably, W11F exhibits an extremely blue-shifted fluorescence (321 nm, when excited at 270 nm); a feature that may be ascribed to a strongly immobilized indole chromophore in a polar environment [27]. Inhibitor binding results in the selective quenching of W168, as demonstrated by the effect of addition of inhibitor (2-phosphoglycolate) on the fluorescence intensity of the two mutant proteins, Fig. 5c [26]. W168 is located on the face of the loop that projects away from the bound ligand. An even more sensitive probe of ligand binding may be generated in an engineered mutant, which has a tryptophan residue on the loop face that is proximal to the bound ligand. Toward this end, we are currently examining a triple mutant of the type W11F/W168F/Y164W.
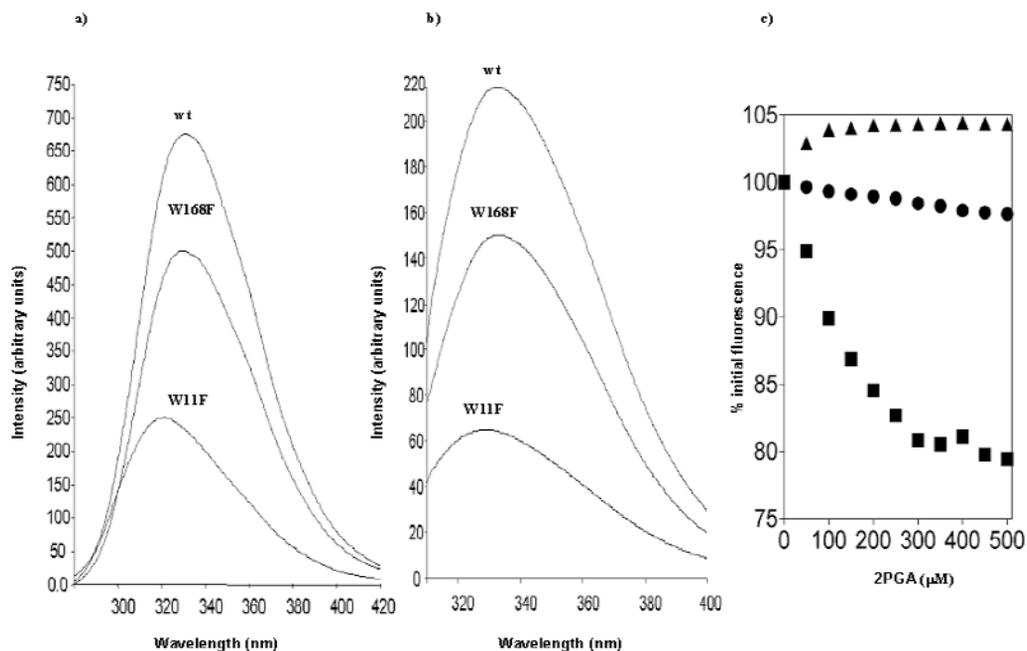
Fig. 5 Steady-state fluorescence emission spectra of PfTIM wild-type (wt) and single tryptophan mutants (W11F and W168F) at pH 8.0. Spectra were recorded after excitation at (a) 270 nm (emission max were 331, 332, and 321 nm for wt, W168F and W11F, respectively); (b) 295 nm (emission max were 331, 332, and 327 nm for wt, W168F and W11F, respectively); (c) Effect of inhibitor binding on fluorescence emission. PfTIM wild-type (=), mutants W11F (<) and W168F (5) were incubated in the presence of increasing concentrations of inhibitor (2PGA). Fluorescence intensities were recorded at the maxima after excitation at 295 nm and plotted as percentage of the intensities in the absence of inhibitor [26].

The high degree of conservation observed for loop 6 residues in available TIM sequences is in accord with the important role ascribed for loop closure in enzyme activity. A schematic view of relationship between loop closure and enzyme activity is summarized in Fig. 6.
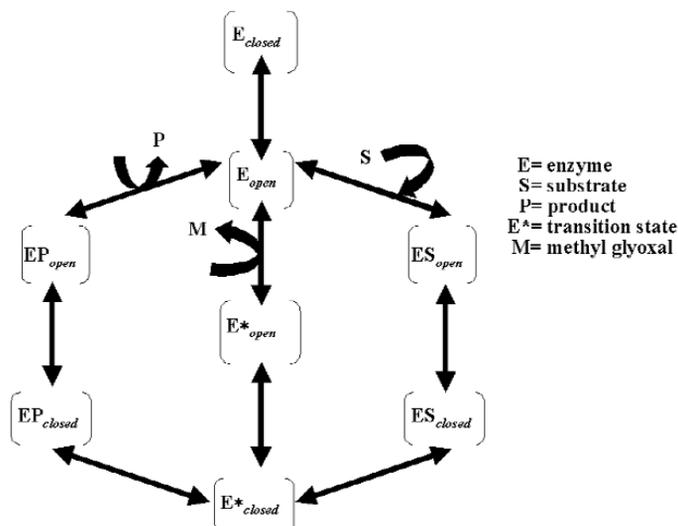
Fig. 6 Schematic representation of loop conformation during catalysis.

STRATEGIES FOR ENZYME INHIBITION

We have explored two distinct approaches to inhibit PfTIM:

•   design of synthetic interface peptides, which can potentially impede subunit association and
•   modification of cysteine (C13), which is proximal to the active site residue lysine (K12).

These strategies have been based on the differences between the human and parasite enzymes at the dimer interface and the absence of C13 in the human enzyme.

Synthetic peptides corresponding to two distinct segments of the subunit interface of the triosephosphate isomerase (residues 9–18, ANWKCNGTLE, peptide I; residues 68–79, KFGNGSYTGEVS, peptide II) were synthesized. A substantial fall in enzyme activity was observed in the presence of peptide II, whereas peptide I did not show any appreciable inhibition (at 1000-fold molar excess of the peptide). The peptide II also showed a more pronounced inhibitory effect on two interface mutants of PfTIM (Y74C and Y74G). The IC_{50} value determined for peptide II was in the range of 0.6–0.8 µM, suggesting that use of interface peptides for inhibition is a viable approach and may also provide lead structures for potential inhibitor design [28].

PfTIM contains four cysteine residues at positions 13, 126, 196, and 217 on each subunit. Of these, Cys-13 is present at the dimer interface and is replaced by methionine in the corresponding human enzyme. Electrospray ionization mass spectrometry coupled with protease-mediated peptide mapping, was used to investigate the effect of sulfhydryl labeling on the parasite enzyme, with a view toward developing selective covalent inhibitors by targeting the interface cysteine residue. Differential labeling of the cysteine residues by iodoacetic acid and iodoacetamide were determined. Iodoacetic acid labeling gave a soluble, largely inactive enzyme, whereas iodoacetamide labeling led to precipitation of the protein. It was also observed that carboxymethylation of Cys-13 results in the formation of monomeric species. An engineered C13D mutant resembling the iodoacetic acid-modified TIM at the interface was also analyzed. The C13D mutant exhibited reduced stability to denaturants and 7-fold reduction in enzymatic activity [29]. Utilization of this reactive Cys at the interface to inhibit TIM from other parasitic organisms is well documented in the literature [30–31].

As mentioned earlier, some of the residues conserved in other TIMs are different in PfTIM and hence may be exploited for targeting. A charged residue, glutamate (E183) at position 183 in human TIM is replaced with a hydrophobic residue leucine in PfTIM, and this region is solvent exposed. Hence, in PfTIM a hydrophobic patch is exposed at a position juxtaposed to a positively charged region. This site was proposed to be specifically suited for inhibitory ligands like hydrophobic anionic molecules. This residue lies close to the active site of PfTIM. Recently, Joubart et al. [32], using the knowledge of the crystal structure and the DOCK algorithm, have identified some lead molecules which inhibit the enzyme, with IC_{50} values <100 µM. Similarly, other important sites like F96 and S73 may be specifically utilized in inhibiting PfTIM.

CONCLUSIONS

The availability of the crystal structure of *P. falciparum* triosephosphate isomerase and several protein inhibitor complexes has raised new questions about the role of loop dynamics in enzyme activity. Critical difference between the parasite and the human enzyme suggests possible approaches to the design of new inhibitors, which target either the dimer interface or unique sulfhydryl residues.

ACKNOWLEDGMENTS

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