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# Crystal structure of *Plasmodium falciparum* thioredoxin reductase, a validated drug target

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ABSTRACT

*Plasmodium falciparum* is the vector of the most prevalent and deadly form of malaria, and, among the *Plasmodium* species, it is the one with the highest rate of drug resistance. At the basis of a rational drug design project there is the selection and characterization of suitable target(s). Thioredoxin reductase, the first protection against reactive oxygen species in the erythrocytic phase of the parasite, is essential for its survival. Hence it represents a good target for the design of new anti-malarial active compounds. In this paper we present the first crystal structure of recombinant *P. falciparum* thioredoxin reductase (PfTrxR) at 2.9 Å and discuss its differences with respect to the human orthologue. The most important one resides in the dimer interface, which offers a good binding site for selective non competitive inhibitors. The striking conservation of this feature among the *Plasmodium* parasites, but not among other Apicomplexa parasites neither in mammals, boosts its exploitability.



## 1. Introduction

Malaria, one of the major threats for human health worldwide, is endemic in more than 100 countries, mainly in tropical and subtropical areas, and causes about 1 million human deaths per year. It represents a huge socioeconomic burden in developing countries, where limited access to therapy has also the drawback of increasing the reservoir of infectivity [1].

Malaria is a vector borne disease caused by the unicellular Apicomplexan parasite belonging to the genus *Plasmodium*. There are four main species infecting humans, and among them *Plasmodium falciparum* is the most deadly and also the one with the highest rate of drug resistance [1].

*Plasmodium* has a complex developmental cycle, which includes a continuous expansion inside host erythrocytes. Therefore it is exposed to high fluxes of reactive oxygen species (ROS), while maintaining a reduced intracellular environment. As a defense mechanism, the parasite evolved a complex network of NADPH-dependent redox enzymes: a complete glutathione (GSH) system and a specialized thioredoxin (Trx) system. The first one comprises GSH, GSH reductase, glutaredoxin (Grx) and Grx-like proteins, GSH-S-transferase (GST), gamma-glutamylcysteine synthetase (gamma-GCS), and a GSH-dependent glyoxalase system. The second system includes Trx reductase (TrxR), several Trxs and Trx-like proteins, and Trx-dependent peroxidases [2–4]. In addition *Plasmodium* can hijack redox proteins from the host. Overall, this proved a winning strategy for survival, despite the lack of catalase and glutathione peroxidase [5].

Using genetic and chemical tools, it was demonstrated that TrxR, the first step of the thioredoxin redox cycle, and gamma-GCS, the rate-limiting step of glutathione synthesis, are essential for parasite survival [3,6]. Therefore some of the enzymes involved in this antioxidant defense pattern represent good targets for the design of new anti-malarial active compounds [7,8]. Indeed PfTrxR is a particularly attractive candidate because of its structural and functional peculiarities: namely, the presence of an extension at the N-terminus and the lack of a selenocysteine in the redox centre at the C-terminus. This last feature is common to all the Apicomplexan TrxRs, where a CGGGKCG stretch replace the GCUC stretch of mammalian TrxRs [9].

Basic to a rational drug design research project there is a thorough functional and structural characterization of a selected target that should display unique features for specificity. In this paper we

*Abbreviations:* PfTrxR, *Plasmodium falciparum* thioredoxin reductase; HsTrxR, homo sapiens thioredoxin reductase; Trx, thioredoxin; GSH, reduced glutathione; NADPH, nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); Tris/HCl, Tris(hydroxymethyl)aminomethane hydrochloride.

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present the first crystal structure of *P. falciparum* thioredoxin reductase (PfTrxR) at 2.9 Å, and discuss its properties in light of the similarities/differences with respect to human TrxR (HsTrxR, PDB ID: 2ZZC [10]), and to computer models of PfTrxR [11,12]. The crystallographic structure unveiled some significant peculiarities of the *Plasmodium* enzyme, which are discussed in terms of their exploitability for drug design.

## 2. Materials and methods

### 2.1. Expression and purification

The gene corresponding to PfTrxR (Primary accession number Q25861) was synthesized and optimized for expression in *Escherichia. coli* cells by GeneArt (Life Technologies), and was cloned into pGEX-4T-1 (GE Healthcare) expression vector, *via* BamHI and XhoI restriction sites.

The protein fused to GST-tag was expressed in BL21(DE3) bacterial cells upon induction with 0.5 mM IPTG, 20 μM FAD, incubating overnight at 16 °C. Cells were lysed by sonication in lysis buffer [0.1 M Tris/HCl, 0.4 M NaCl, pH 7.4, 5 mM β-mercaptoethanol, 5% glycerol, 10 μg/ml DNase, a cocktail of protease inhibitors (COMPLETE, Roche) and 2 mM EDTA]. The protein was purified from the soluble fraction by affinity chromatography on Glutathione Sepharose (QIAGEN). Successful expression of PfTrxR was confirmed by SDS–PAGE, highlighting a band at approximately 86 kDa (Figure not shown). The GST-tag was cleaved by thrombin (Sigma–Aldrich) that was finally removed passing the fractions on a 1 ml benzamidine FF (HS) column (GE Healthcare). The expression gave a yield of 7 mg/L culture, with a good FAD incorporation, according to the $Abs_{280}/Abs_{340}$ ratio (data not shown).

Purified PfTrxR was exchanged into crystallization buffer (25 mM Tris/HCl, 0.2 M NaCl, pH 7.4, 5 mM β-mercaptoethanol), and concentrated to 3.5 mg/mL by ultrafiltration (Millipore), aliquoted, and stored at −20 °C.

### 2.2. Crystallization

Crystallization conditions were initially screened by robot (Phoenix, ArtRobbins) and then optimized by standard hanging drop methods. Crystals of PfTrxR grew over 1 month in a drop composed by 1 μl protein (3.5 mg/mL) and 1 μl well solution (16% (w/v) PEG 4000, 0.1 M Tris/HCl, pH 8.5, 0.2 M sodium acetate).

### 2.3. Data collection, processing, and refinement

Diffraction data have been collected on BL14.1 (HZB BESSY II electron storage ring of Berlin-Adlershof, Germany [13]) from a crystal diffracting up to 2.9 Å. Data were indexed and processed with XDS [14]. Phases were determined by molecular replacement using Phaser [15] within the CCP4 Suite [16,17], taking mitochondrial mouse TrxR as a model (PDB entry: 1ZKQ, 46% identity [18]).

The structure was refined using REFMAC5 [19] and fitted to generated electron density maps by Coot [20]. MolProbity [21] and ProCheck [22] were used to assess the quality of the final model. Data collection and refinement statistics are summarized in Table 1.

Figures were prepared with CCP4MG [23]. All the sequence alignments were made with ClustalW2 [24] available on the EBI website (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

### 2.4. Functional assays

#### 2.4.1. DTNB reduction assay

5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) reduction assay was performed by adding PfTrxR (5–500 nM) to a mixture of 100 mM potassium phosphate pH 7.0, 2 mM EDTA, 0.2–5.0 mM DTNB, and 300 μM NADPH at 20 °C. The increase in absorbance at 412 nm was monitored ($\varepsilon_{412}$ = 13.6 $mM^{-1}$ $cm^{-1}$) [25].

**Table 1**
Summary of data collection and refinement statistics for 4B1B.

| | |
|---|---|
| *Data collection* | |
| Space group | $P2_12_12_1$ |
| Cell dimensions: *a*, *b*, *c* (Å) | 63.12, 109.18, 182.39 |
| Resolution range (Å) (last shell) | 15.7–2.9 (3.07–2.90) |
| $R_{merge}$ (last shell) | 0.19 (0.76) |
| $I/\sigma I$ (last shell) | 9.23 (2.02) |
| Completeness (%) | 96.7 (92.7) |
| Multiplicity | 5.1 (4.4) |
| No. total reflections | 137765 |
| No. unique reflections | 27908 |
| *Refinement* | |
| Resolution (Å) | 15.7–2.9 |
| No. reflections used | 26474 |
| R factor | 0.26 |
| $R_{free}$ | 0.29 |
| No. atoms | 6848 |
| Protein | 6739 |
| Ligand | 109 |
| *B*-Wilson ($Å^2$) | 52.2 |
| *B*-factor ($Å^2$) | 34.25 |
| *Model quality* | |
| R.m.s deviations | |
| Bond lengths (Å) | 0.002 |
| Bond angles (°) | 0.39 |
| Chiral volume ($Å^2$) | 0.028 |
| Ramachandran plot (%) | |
| Favored | 99.7 |
| Generously allowed | 0.3 |
| Disallowed | 0 |

#### 2.4.2. Insulin reduction assay

The classical turbidimetric assay [26] was performed by following the precipitation of insulin at 650 nm after addition of 300 nM PfTrxR to a reaction mixture composed of 100 mM potassium phosphate pH 7.0, 2 mM EDTA, 300 μM NADPH, 200 μM insulin, and 10 μM thioredoxin from *Schistosoma mansoni*. Stock solutions of insulin were prepared according to [26]. The parameters used to express the enzymatic activity were the starting time of precipitation and the precipitation rate ($\Delta Abs_{650}$ $min^{-1}$).

## 3. Results

### 3.1. The crystal structure

PfTrxR crystals belong to space group $P2_12_12_1$, with two monomers per asymmetric unit, in agreement with the dimeric active form of the enzyme [5]. Crystal diffraction at 2.9 Å allowed the resolution of the overall fold, which is superimposable to that of other members of the class-I pyridine nucleotide-disulfide oxidoreductase family. PfTrxR is a homodimeric enzyme, whose monomer has three domains: a NADPH binding domain, a FAD binding domain, and a monomer–monomer interface. It possesses three redox centers: (i) the non covalently bound FAD, which takes up electron from NADPH; (ii) the first di-thiol couple Cys88–Cys93, which takes up electrons from the isoalloxazine ring of FAD; (iii) the second di-thiol couple Cys535–Cys540 at the end of the C-terminal arm. The enzyme is an obligate dimer since the C-term Cys couple of one monomer is in close contact with the FAD Cys couple of the adjacent one, from which it takes up electrons to subsequently reduce the macromolecular substrate Trx.

All the secondary structure elements, the active site in the proximity of FAD (Supplementary material Fig. 1), the NADPH binding