Identification and Characterization of the Functional Amino Acids at the Active Site of the Large Thioredoxin Reductase from *Plasmodium falciparum*

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The thioredoxin system, composed of the pyridine nucleotide-disulfide oxidoreductase thioredoxin reductase, the small peptide thioredoxin, and NADPH as a reducing cofactor, is one of the major thiol-reducing systems of the cell. Recent studies revealed that *Plasmodium falciparum* and human thioredoxin reductase represent a novel class of enzymes, called large thioredoxin reductases. The large thioredoxin reductases are substantially different from the isofunctional prokaryotic *Escherichia coli* enzyme. The putative essential amino acids at the catalytic center of large thioredoxin reductase from *P. falciparum* were determined by using site-directed mutagenesis techniques. To analyze the putative active site cysteines (Cys88 and Cys93) three mutant proteins were constructed substituting alanine or serine residues for cysteine residues. Further, to evaluate the function of His509 as a putative proton donor/acceptor of large thioredoxin reductase this residue was replaced by either glutamine or alanine. All mutants were expressed in the *E. coli* system and characterized. Steady state kinetic analysis revealed that the replacement of Cys88 by either alanine or serine and Cys93 by alanine resulted in a total loss of enzymatic activity. These results clearly identify Cys88 and Cys93 as the active site thiols of large thioredoxin reductase. The replacement of His509 by glutamine yielded in a 95% loss of thioredoxin reductase activity; replacement by alanine provoked a loss of 97% of enzymatic activity. These results identify His509 as active site base, but imply that its function can be substituted, although inefficiently, by an alternative proton donor, similar to glutathione reductase. Spectral analysis of wild-type *P. falciparum* thioredoxin reductase revealed a 550-nm absorption band upon reduction which resembles the EH2 form of glutathione reductase and lipoamide dehydrogenase.

This spectral feature, recently also reported for the human placenta TrxR, further illustrates the similarity between large thioredoxin reductases and glutathione reductases and stresses the profound differences to small *E. coli* thioredoxin reductase.

EXPERIMENTAL PROCEDURES

*Mutagenic Oligonucleotides and Site-directed Mutagenesis—Ten oligonucleotides were designed to replace amino acid residues potentially involved in *P. falciparum* TrxR activity (Table I). The putative active site cysteines, Cys88 and Cys93, were changed to alanine. Residue Cys93 was also replaced by either glutamine or alanine.*

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*The abbreviations used are: TrxR, thioredoxin reductase; PfTrxR, *Plasmodium falciparum* TrxR; PTrxR/C88A, TrxR containing alanine at position 88 instead of cysteine; PTrxR/C88S, PTrxR containing serine at position 88 instead of cysteine; PTrxR/C83A, PTrxR containing alanine at position 83 instead of cysteine; PTrxR/H509A, PTrxR containing alanine at position 509 instead of histidine; PTrxR/H509Q, PTrxR containing glutamine at position 509 instead of histidine; DTNB, 5,5′-dithiobis (2-nitrobenzoate).*

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and sonicated (Branson Sonifier 250) in binding buffer (20 mM Tris/HCl, pH 7.9). For spectral analyses of *E. coli* TRX RC88A at various pH values, the protein concentration was determined since the discrimination in rate could not be achieved even at a concentration of NADPH as low as 2 μM.

The kinetic parameters of wild-type *Pf* TrxR were previously reported that the Km of wild-type and mutant *Pf* TrxRs were determined by the DTNB reduction assay.

| TABLE I | Mutagenic oligonucleotides for site-directed mutagenesis of PfTrxR |
|---|---|
| Nucleotides that were exchanged in order to obtain the desired mutation are underlined and in bold letters. Numbers in superscript indicate the position of nucleotides in the coding region of the gene. |
| 5′*247*CGATTCAGCATCTGTTGGA 3′ | C88WT<sup>a</sup> |
| 5′*247*CGATTCAGCATCTGTTGG 3′ | C88A (S)<sup>b</sup> |
| 5′*247*CGATTCAGCATCTGTTGG 3′ | C88A (AS)<sup>c</sup> |
| 5′*265*GTGAACGTAGGATGTGTACC 3′ | C93A (S) |
| 5′*265*GTGAACGTAGGATGTGTACC 3′ | C93A (AS) |

<sup>a</sup> WT, wild type.

<sup>b</sup> S, sense.

<sup>c</sup> AS, antisense.

changed to serine. The putative active site base His<sup>299</sup> was replaced by either alanine or glutamine. The replacement of the original amino acids with the desired ones was achieved by an in vitro site-directed mutagenesis method according to Papworth et al. (14). By using high fidelity thermostable *Pfu* DNA polymerase, low cycle number, and primers designed only to copy the parental strand in a linear fashion, this method minimized unwanted second site mutations and generated mutants in a 1-day procedure. Briefly, the mutagenic oligonucleotides, complementary to the opposite strand of the double-stranded DNA template, were extended by the *Pfu* DNA polymerase during temperature cycling. 35 ng of the double-stranded, supercoiled expression plasmids were used for mutagenesis and protein expression. The mutagenic oligonucleotides, reaction buffer, and *Pfu* DNA polymerase according to the manufacturer’s recommendations (Stratagene). The cycling parameters were 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 8 min, 12 cycles. The linear amplification product was treated with endonuclease *Dpn* I (10 units/μl, Boehringer Mannheim) for 1 h to eliminate the parental template. Subsequently, an aliquot of 6 μl of this reaction mixture containing the double-nicked mutated plasmid was used for the transformation of competent *E. coli* BL 21 (DE3) cells (Stratagene). All mutants were analyzed performing the Sanger dideoxy chain termination reaction for the position of nucleotides in the coding region of the gene.

**Expression and Purification of Mutant Thioredoxin Reductase**—The expression plasmid pJC40/PfTrxR (5) and 100 ng of mutagenic sense and antisense primers were used in a 50-μl reaction mixture containing deoxyribonucleotides, reaction buffer, and *Pfu* DNA polymerase according to the manufacturer’s recommendations (Stratagene). The cycling parameters were 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 8 min, 12 cycles. The linear amplification product was treated with endonuclease *Dpn* I (10 units/μl, Boehringer Mannheim) for 1 h to eliminate the parental template. Subsequently, an aliquot of 6 μl of this reaction mixture containing the double-nicked mutated plasmid was used for the transformation of competent *E. coli* BL 21 (DE3) cells (Stratagene). All mutants were analyzed performing the Sanger dideoxy chain termination reaction for double-stranded DNA (15). 100% of the analyzed colonies contained the analyzed performing the Sanger dideoxy chain termination reaction for double-stranded DNA (15). 100% of the analyzed colonies contained the desired mutation in the absence of other unwanted mutations.

**Expression and Purification of Mutant Thioredoxin Reductase**—The expression plasmid pJC40 (16), which contains a T7 promoter and encodes 10 histidine residues preceding the N terminus of the recombinant protein, was used for overexpression of the wild-type *Pf* TrxR and all mutants in *E. coli* BL 21 (DE3) cells. All mutants were analyzed performing the Sanger dideoxy chain termination reaction for double-stranded DNA (15). 100% of the analyzed colonies contained the desired mutation in the absence of other unwanted mutations.

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compared based upon their kinetic properties (Table II). To evaluate the role of Cys88, Cys93, and His509 as putative catalytic active residues, the DTNB reduction assay was performed with wild-type and mutant proteins. Mutation of either Cys88 or Cys93 to alanine or serine resulted in a total loss of DTNB reducing wild-type PfTrxR. Replacement of His509 by either glutamine or alanine drastically decreased the reduction capacity of PfTrxR which implies that His509 represents the active site base of PfTrxR. PfTrxRH509Q retained about 5% of residual activity toward DTNB compared with the wild-type protein. To eliminate the possibility of a partial compensation of the imidazole side chain by the amido group of glutamine, we substituted His909 by alanine. However, the H509A mutant still retained 3% of the wild-type enzyme activity with DTNB. Although the turnover number of the histidine mutants decreased drastically, these mutants had a lower Km value for the model substrate DTNB (Table II). Additionally, the Km value for NADPH of the H509A mutant decreased. A similar effect on the Km value for NADPH is well known for the same point mutation of Cys88 in E. coli TrxR by a shift of 21 nm toward the blue (20). A permanent charge transfer complex is inducible at pH values higher than 8.5. A permanent charge transfer complex is formed even in the absence of a reductant under aerobic conditions. The absorption spectrum at pH 7.9 (original conditions) and pH 10.6. Apart from the induction of a long wavelength band around 580 nm, the pH change from 7.9 to 10.6 resulted in a slight increase of the absorbance at 550 nm and a slight increase of the absorption at the 550-nm band. The exchange of the Cys88 with serine has drastic effects on the enzyme as expected if the charge transfer band is due to interaction of Cys88 with the protein-bound flavin. The purified protein has a brick-red color instead of being yellow as all other proteins. Spectral analysis of nonreduced PfTrxRC88A shows the presence of a charge transfer complex with a λmax at 442 nm and a shoulder around 580 nm (Fig. 4), resembling the charge transfer produced in the EHL species of glutathione reductase and trypanothione reductase (19, 21). Remarkably, PfTrxRC88A is yellow and shows only a slight increase of the absorbance at 550 nm compared with the oxidized wild-type protein, and addition of NADPH does not change the spectral features apart from bleaching the absorbance at 451 nm due to FAD reduction. Determination of the oxidized spectra of PfTrxRC88A at higher pH values resulted in spectral changes of the nonreduced protein. Fig. 2B shows the absorption spectrum at pH 7.9 (original conditions) and pH 10.6. Apart from the induction of a long wavelength band around 580 nm, the pH change from 7.9 to 10.6 resulted in a blue shift of the flavin peak by 21 nm. The appearance of the 580-nm band at higher pH values suggests that the alanine mutation of Cys88 affects the pK of Cys88 so that this residue is not in its thiolate form at pH values lower than 8.5 (Fig. 2C) and can thus not interact with the protein-bound flavin of

**FIG. 1. Spectra of wild-type PfTrxR**

| Enzyme | λmax (nm) | Absorption coefficient | Charge transfer complex |
|--------|-----------|-----------------------|------------------------|
| WT     | 462       | 11.43 ± 0.95          | +                     |
| C88A   | 451       | 12.80 ± 1.40          | –                     |
| C88S   | 442       | 12.00 ± 0.73          | +                     |
| C93A   | 456       | 12.62 ± 1.20          | –                     |
| H509A  | 462       | 13.58 ± 1.47          | –                     |
| H509Q  | 460       | 11.40 ± 0.34          | –                     |

* A distinct shoulder with the λmax at 550 nm is formed under anaerobic conditions in the presence of 10 equivalents of NADPH/FAD.

* No charge transfer complex at pH 7.9. A permanent charge transfer complex is inducible at pH values higher than 8.5.

* A permanent charge transfer complex is formed even in the absence of a reductant under aerobic conditions.

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FIG. 2. A, spectra of PfTrxRC88A (in 20 mM Tris/HCl containing 0.5 M NaCl and 1 mM EDTA, pH 7.9): a, oxidized PfTrxRC88A (10 μM); b, reduced PfTrxRC88A. Reduction was achieved by addition of 10 equivalents of NADPH/FAD under anaerobic conditions. B, spectra of PfTrxRC88A at various pH values. a, oxidized PfTrxRC88A (8.8 μM) in 20 mM Tris/HCl, containing 0.5 M NaCl, pH 7.9. b, oxidized PfTrxRC88A (9.5 μM) in 200 mM glycine NaOH containing 0.5 M NaCl, pH 10.6. C, details of oxidized spectra of PfTrxRC88A between 500 and 800 nm using different buffer systems. 1 and 2, 20 mM Tris/HCl containing 0.5 M NaCl and 0.1 mM EDTA, pH 8.5 and 8.9, respectively. 3–5, 200 mM glycine NaOH containing 0.5 M NaCl and 0.1 mM EDTA at pH 9.5, 10.0, and 10.6, respectively.
Further, these data imply that Cys93 represents the charge transfer thiol of PfTrxR.

**DISCUSSION**

Large thioredoxin-reductases represented by the human, bovine and *P. falciparum* proteins differ considerably from the small *E. coli* TrxR (5, 6, 8, 11, 18). These two types of isofunctional enzymes are distinct in molecular mass, location of their active site residues, and most likely by their structure and catalytic mechanism (5, 6, 8). Molecular analyses of PfTrxR and the human enzyme revealed a high degree of similarity of both proteins to glutathione reductases rather than to *E. coli* TrxR, which is further supported by the results of Arscott et al. (8) for human TrxR and the data presented here for PfTrxR.

A series of PfTrxR mutants altered at the potential redox-active disulfide bridge (Cys88 and Cys93) and the potential active site base (His509) were generated to obtain more information about their role for the catalytic reaction of large TrxRs. The conversion of either one of the putative active site thiols, Cys88 and Cys93, into alanine or serine caused a complete inactivation of the thiol-reducing ability of PfTrxR. These data clearly identify Cys88 and Cys93 as essential amino acid residues involved in the catalysis of PfTrxR. Similar results were obtained when isofunctional residues were mutated in trypanothione reductase and glutathione reductase (19, 22). In contrast the replacement of the isofunctional residues in *E. coli* TrxR resulted in a residual activity of the mutant proteins of about 10%, which was explained by the different topology of the protein-bound flavin and the disulfide axis in comparison to other members of this protein family (11).

Further, it was demonstrated that His509 is involved in the catalytic mechanism of PfTrxR. The catalytic activity toward DTNB was drastically reduced but not completely eliminated with the substitution of the His509 by either glutamine or alanine. The residual activity of 5% after substitution of His509 by glutamine might be due to the hydrogen-bonding capacity of the glutamine residue or to the participation of another protonable side chain in the enzyme. To completely remove the potential hydrogen-bonding capacity, His509 was replaced by alanine. PfTrxRH509A still maintained 3% of catalytic activity with DTNB. This result implies that the function of His509 can be substituted, although inefficiently, by a nearby side chain that functions as an alternative proton acceptor/donor. These data are consistent with investigations on the *E. coli* glutathione reductase, where the analogous mutants H439Q and
H439A retained 1 and 0.3%, respectively, of the activity of the wild-type enzyme (20). In contrast, in E. coli TrxR Asp139 rather than His245 functions as the acid catalyst for the dithiol-disulfide redox interconversion (23).

The most characteristic spectral feature of wild-type PfTrxR is the formation of a new 550-nm absorption band upon NADPH reduction (Fig. 1). This long wavelength absorbance resembles other EH2 forms of related proteins (19, 21, 24). It is indicative of a thiolate flavin charge transfer complex as a stable intermediate of the two electron reduced form of the enzyme (25). Recently, the formation of a thiolate flavin charge transfer complex upon reduction with NADPH and dithionite was described for human TrxR (8) but does not occur in the E. coli enzyme (11, 26). The absorption spectrum of EH2 is influenced by complex formation with NADP+ and/or NADPH (27). Such a modulation might also occur in the spectrum reported here for the NADPH-reduced wild-type PfTrxR slightly increasing the absorbance around 550 nm.

To delineate the role of the active site cysteines and to identify the residue responsible for the thiolate flavin charge transfer interaction of EH2, we exchanged the putative active site cysteine of PfTrxR. In related flavoproteins such as glutathione reductase, trypanothione reductase, and mercuric ion reductase, the carbonyl-terminal cysteine residue of the redox active site is responsible for the formation of the thiolate flavin charge transfer complex (19, 24, 28). The replacement of Cys93 by alanine (PfTrxRC93A) does not change the spectral features of the oxidized form of the protein. Upon reduction with an excess of NADPH a slight increase of the 550-nm band occurs (Fig. 3). This might be due to the binding of excess NADPH to the reduced protein. The replacement of the other active site cysteine Cys88 resulted in conspicuous spectral properties. While a serine at this position leads to the expected distinctive absorption spectrum of the oxidized protein resembling a peroxidase (29), the amino-terminal cysteine residue sufficiently perturbs the environment of the charge transfer donor and might increase its pK (21). However, in this case binding of 3-aminopyridine adenine dinucleotide to pig heart lipoamide dehydrogenase induces the appearance of the long wavelength band and is assigned to a charge transfer complex between the carbonyl-terminal thiolate and the protein-bound flavin (30). In glutathione reductase monoalkylated at the amino-terminal cysteine residue, the long wavelength band is fully retained (21). Decreasing the pH value to 3.5 results in almost a complete loss of the charge transfer absorbance; increasing the pH to 10.3 yields a blue shift of the FAD band and increases the long wavelength absorbance (31), also seen in PfTrxRC88A (Fig. 2B).

In conclusion, the data presented here suggest fundamental differences between small TrxRs and large TrxRs. Cys88, Cys93, and His509 were identified as essential active site residues of PfTrxR. Equivalent residues are responsible for the enzymatic activity of glutathione reductase, whereas in E. coli TrxR the active site residues show a completely different topology (9, 11). Further, the formation of a stable thiolate flavin charge transfer complex implies that the reaction mechanism of large TrxRs is similar to glutathione reductase and lipoamide dehydrogenase as it has also been suggested by Arscott et al. (8). However, the fact that PfTrxRC88A does not show the expected charge transfer band at pH values lower than 8.5 hints toward distinctive differences in the architecture of the catalytic center of glutathione reductase and PfTrxR, that demand more detailed studies dealing with the acid-base chemistry of the PfTrxR active site. In addition, crystallographic analysis of the threedimensional structure will be necessary to conclusively discuss the active site geometry of the protein.

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