The role of the C-terminus for catalysis of the large thioredoxin reductase from *Plasmodium falciparum*

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Abstract The thioredoxin system is one of the major thiol reducing systems of the cell. Recent studies have revealed that *Plasmodium falciparum* and human thioredoxin reductase represent a novel class of enzymes, which are substantially different from the isofunctional prokaryotic *Escherichia coli* enzyme. We identified the cysteines Cys^{88} and Cys^{93} as the redox active disulfide and His^{509} as the active site base [Gilberger, T.-W., Walter, R.D. and Müller, S., J. Biol. Chem. 272 (1997) 29584–29589]. In addition to the active site thiols Cys^{88} and Cys^{93} the *P. falciparum* enzyme has another pair of cysteines at the C-terminus: Cys^{535} and Cys^{540}. To assess the possible role of these peripheral cysteines in the catalytic process the single mutants *Pf*TrxRC535A and *Pf*TrxRC540A, the double mutant *Pf*TrxRC535AC540A and the deletion mutant *Pf*TrxRΔ9 (C-terminal deletion of the last nine amino acids) were constructed. All mutants are defective in their thioredoxin reduction activity, although they still show reactivity with 5,5′-dithiobis (2-nitrobenzoate). These data imply that the C-terminal cysteines are crucially involved in substrate coordination and/or electron transfer during reduction of the peptide substrate.

Key words: Large thioredoxin reductase; C-terminal residue; Site-directed mutagenesis; *Plasmodium falciparum*

1. Introduction

Thioredoxin reductase (TrxR) is a homodimeric protein which belongs to the family of pyridine nucleotide-disulfide oxidoreductases like lipoamide dehydrogenase and glutathione reductase (GR) [1]. In general the members of this group of proteins show a high degree of similarity at the molecular and mechanistic levels irrespective of their source. However, this does not apply to TrxRs, which can be separated into at least two classes: low M_r and high M_r TrxRs. These proteins differ not only in their primary structures but also in their subunit molecular mass and their proposed reaction mechanisms [2–5]. The *Escherichia coli* TrxR is a representative of the low M_r TrxRs and its reaction mechanism has been investigated in great detail [6–8]. In order to react with its substrate the enzyme has to undergo a conformational change. The reaction mechanism of high M_r TrxRs is completely different from that of low M_r TrxRs and resembles that of glutathione reductase and lipoamide dehydrogenase [4,5]. Remarkably, the proteins from human placenta, lung adenocarcinoma cells and human T-cells possess a penultimate C-terminal selenocysteine residue (SeCys) adjacent to a cysteine residue [9–11] which represents an additional potentially redox-active component in the mammalian TrxRs [4]. The *Plasmodium falciparum* protein, however, does not contain a SeCys but possesses a C-terminal extension (Table 1), including two cysteine residues which are separated by four amino acids rather than being adjacent, as found in the mammalian TrxRs. The occurrence of a C-terminal cysteine pair is reminiscent of another member of the flavin disulfide oxidoreductase family, the mercuric ion reductases [12] (Table 1). It has been shown that these residues (Cys^{558} and Cys^{559}) are essential for a high level of mercuric ion reductase activity [12–14].

Considering these facts the *Pf*TrxR with its extended C-terminus and the terminal cysteine residues Cys^{535} and Cys^{540} suggests the possibility of an involvement of these peripheral amino acids in the catalytic mechanism of this high M_r TrxR. To elucidate the role of Cys^{535} and Cys^{540} for catalysis of *Pf*TrxR we performed mutagenesis studies and characterized the recombinantly expressed *Pf*TrxR mutant proteins.

2. Materials and methods

2.1. Mutagenic oligonucleotides and site-directed mutagenesis

The mutagenic oligonucleotides used to generate the mutant constructs are shown in Table 2. Two different in vitro mutagenesis methods were applied. The site-directed exchange of amino acids was performed as previously described [5,15]. All mutant constructs were analyzed performing the Sanger dideoxy chain termination reaction for double stranded DNA [16].

The C-terminal deletion mutant was generated by PCR using *Pfu* DNA polymerase (Stratagene). The antisense oligonucleotide Δ9 was designed with a *Bam*HI restriction site resulting in a transcription termination at the respective site of the coding region of *Pf*TrxR (Table 2). The 5′ sense oligonucleotide contained a *Hin*dIII restriction site and encodes the first eight amino acids of *Pf*TrxR (Table 2). The PCR (50 μl) contained 50 ng of the double stranded, supercoiled expression plasmid pJC40*Pf*TrxR [3] and 100 ng mutagenic sense and antisense primer, deoxyribonucleotides, reaction buffer and *Pfu* DNA polymerase according to the manufacturer's recommendations (Stratagene). PCR used the following protocol: 95°C (1 min) following 30 cycles of 95°C (30 s), 55°C (1 min), 68°C (3 min). The isolated PCR product was restricted with *Bam*HI and *Hin*dIII and subcloned into pJC20 [17], previously digested with *Bam*HI and *Hin*dIII (Boehringer Mannheim). The ligation product was used to transform *E. coli* BL 21 (DE3). The mutant was analyzed performing the Sanger dideoxy chain termination reaction for double stranded DNA [16].

2.2. Expression and purification of P. falciparum thioredoxin reductases

The expression plasmid pJC20 [17] which contains a T7 promoter was used for overexpression of the wild-type *Pf*TrxR and all mutants

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Abbreviations: DTNB, 5,5′-dithiobis (2-nitrobenzoate); TrxR, thioredoxin reductase; *Pf*TrxR, *Plasmodium falciparum* TrxR; *Pf*TrxRC535A, *Pf*TrxR containing alanine at position 535 instead of cysteine; *Pf*TrxRC540A, *Pf*TrxR containing alanine at position 540 instead of cysteine; *Pf*TrxRC535AC540A, *Pf*TrxR containing alanines at positions 535 and 540 instead of cysteines; *Pf*TrxRΔ9, *Pf*TrxR without the last nine C-terminal amino acids

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in *E. coli* BL21 (DE3) cells. *E. coli* BL 21 (DE3) cells carrying the expression plasmid containing the mutated cDNA were grown overnight at 37°C in Luria-Bertani medium (containing 50 μg/ml ampicillin). Expression was carried out in a 2 liter high density fermenter. 300 ml of the overnight culture was added to 1.2 liter of terrific broth medium [16]. The cultures were grown at 37°C to an OD_{600} of 3, subsequently 500 ml of terrific broth medium, 50 μg/ml ampicillin and 1 mM isopropyl β-D-thiogalactoside were added to the cultures. The cells were harvested 3 h after induction by centrifugation ($4000\times g$; 10 min) and sonicated (Sonifier 250, Branson) in 50 mM potassium phosphate buffer pH 7.6 containing 1 mM EDTA (buffer A). The recombinant proteins were purified as previously described [18,19], with modifications. The cell lysate was centrifuged at $100\,000\times g$ for 1 h (Centrikon 1065, Kontron). The resulting supernatant was saturated to 40% with ammonium sulfate, the precipitated protein removed by centrifugation and overexpressed *Pf*TrxR wild-type and mutant proteins were precipitated by saturating the supernatant to 70% ammonium sulfate. The precipitate was dissolved in buffer A and dialyzed against two changes of the same buffer overnight. Subsequently, the protein was applied to 2′,5′-ADP agarose (Sigma) previously equilibrated with buffer A and washed with the same buffer until no more protein was detected in the flow through. Non-specifically bound protein was washed off the resin with buffer A containing 0.1 M KCl and *Pf*TrxR wild-type and mutant proteins were eluted with buffer A containing 0.1 M KCl and 1 mM $NADP^+$. In order to improve the purity of the protein and to remove the $NADP^+$, the fractions with highest 5,5′-dithiobis (2-nitrobenzoate) (DTNB)-reducing activity were pooled and supplied to a Sephadex S-200 FPLC column (Pharmacia, Freiburg) previously equilibrated with buffer A. The purity of the recombinant proteins was assessed by SDS-PAGE.

2.3. Expression and purification of E. coli thioredoxin

E. coli thioredoxin was recombinantly expressed in *E. coli* by using the plasmid pDL59TΔ4 containing the *trxA* gene (*E. coli* thioredoxin) (S.B. Mulrooney, 1997, unpublished) kindly provided by C.H. Williams Jr., Ann Arbor, MI, USA. The peptide was purified according to D. Veine and C.H. Williams Jr. (personal communication). The purity of the recombinant *E. coli* thioredoxin was checked on a 15% SDS-PAGE; for quantification the extinction at 280 nm ($13\,700\ M^{-1}\ cm^{-1}$) was used.

2.4. FAD contents and extinction coefficients

The extinction coefficients of protein bound FAD in the 450 nm region were determined for all *Pf*TrxRs as previously described [5,20]. Values are means of three determinations and were used for protein quantification: *Pf*TrxR $12.49\pm0.34\ mM^{-1}\ cm^{-1}$, *Pf*TrxRC535A $12.04\pm0.52\ mM^{-1}\ cm^{-1}$, *Pf*TrxRC540A $13.55\pm0.30\ mM^{-1}\ cm^{-1}$, *Pf*TrxRC535AC540A $12.30\pm0.22\ mM^{-1}\ cm^{-1}$, *Pf*TrxRΔ9 $12.29\pm0.08\ mM^{-1}\ cm^{-1}$.

2.5. Spectral analysis

Oxidized and NADPH reduced absorption spectra of wild-type and *Pf*TrxRC535AC540A were recorded in a thermostatted spectrophotometer (Uvikon 932, Kontron) at 22°C as previously described [5].

Table 1
C-terminal amino acid sequences of various TrxRs and mercuric ion reductase

		Reference
*Pf*TrxR	^{530}AAKGGCGGGKC G Stop	[3,23]
*Hs*TrxR	^{488}SGASILQAGCU*G Stop	[2,10]
CeTrxR	^{488}SGQDPRTQGCC G Stop	[24]
*Pa*MerA	^{555}NKDVKQLSCC AG Stop	[25]
*Ec*TrxR	^{310}AERYLDGLADA K Stop	[26]

U* represents SeCys.

2.6. Thioredoxin reductase activity

The kinetic parameters of wild-type *Pf*TrxR and all mutant proteins were determined by the DTNB reduction assay and the thioredoxin reduction assay [3,18,21] using *E. coli* thioredoxin (Trx-S_2) as a substrate. The thioredoxin reduction assay mixture (1 ml) contained 100 mM HEPES-NaOH buffer, pH 7.6, 2 mM EDTA, 200 μM NADPH, 80 μM *E. coli* Trx-S_2, 0.2 mg bovine serum albumin, 0.4 mg human insulin (Sigma) and 1–3 μg enzyme. Assay mixtures lacking Trx-S_2 served as controls. The change in absorbance at 340 nm was monitored in a thermostatted spectrophotometer at 37°C (Uvikon 932, Kontron).

3. Results

3.1. Enzyme purification

It was previously reported that *Pf*TrxR can be expressed as an active His-tag fusion homodimer in the *E. coli* system [3]. Here we report the expression and purification of non-fusion wild-type enzyme and C-terminal mutants. Using the expression and purification scheme described above the yield of recombinant non-fusion proteins was in the range of 4–10 mg pure protein per liter of bacterial culture for the wild-type and mutant TrxRs. The homogeneity of all proteins was assessed by SDS-PAGE (data not shown).

3.2. Kinetics of the PfTrxR and mutated enzymes

Reductase activity of wild-type enzyme and mutants was determined with DTNB and *E. coli* Trx-S_2. The apparent kinetic parameters of all enzymes are summarized in Table 3. The modification of the C-terminus had a significant effect on the catalytic activity towards DTNB. The K_m values of both *Pf*TrxRC535A and *Pf*TrxRC540A increased about 3-fold, whereas the specific activities are comparable to that of the wild-type protein. The double mutation *Pf*TrxRC535A-C540A raised the K_m for this substrate by a factor of 8, similar to the changes observed for *Pf*TrxRΔ9, the C-terminal

Table 2
Mutagenic oligonucleotides for site-directed mutagenesis of *Pf*TrxR

5′ ^{1590}GCTAAAGGAGGATGTGGGGGTGGAAAATGTGG 3′	WT
5′ GCTAAAGGAGGA**GC**TGGGGGTGGAAAATGTGG 3′	C535A (S)*
5′ CCACATTTTCCACCCCCA**GC**TCCTCCTTTAGC 3′	C535A (AS)*
5′ ^{1605}GGGGGTGGAAAATGTGGA*TAA*GGATCCCGGG 3′	WT
5′ . . .GGGGGTGGAAAA**GC**TGGATAA*GGATC*CCGGG 3′	C540A (S)
5′ . . .CCCGGG*ATCC*TTATCCA**GC**TTTTCCACCCCC 3′	C540A (AS)
5′ ^{1590}GCTAAAGGAGGATGTGGGGGTGGAAAATGTGGA*TAA*GGATCCCCG 3′	WT
5′ GCTAAAGGAGGA**GC**TGGGGGTGGAAAAT**GC**GGA*TAA*GGATCCCCG 3′	C535AC540A(S)
5′ CGGGGATCCTTATCCA**GC**TTTTCCACCCCCA**GC**TCCTCCTTTAGC 3′	C535AC540A(AS)
5′ GCGC*AAGCTT*ATGTGTAAAGATAAAAACGAAAAAAAAAATTATG 3′	ExHind (S)
5′ GCGC*GGATCC*TTATTTAGCTGCATAAGACAATCC 3′	Δ9 (AS)

Nucleotides that were exchanged in order to obtain the desired mutation are in bold letters. Nucleotides underlined represent start and stop codon, respectively. Nucleotides in italics represent restriction sites. Numbers in superscript indicate the position of nucleotides in the coding region of the gene. WT, wild-type, (S), sense; (AS), antisense.

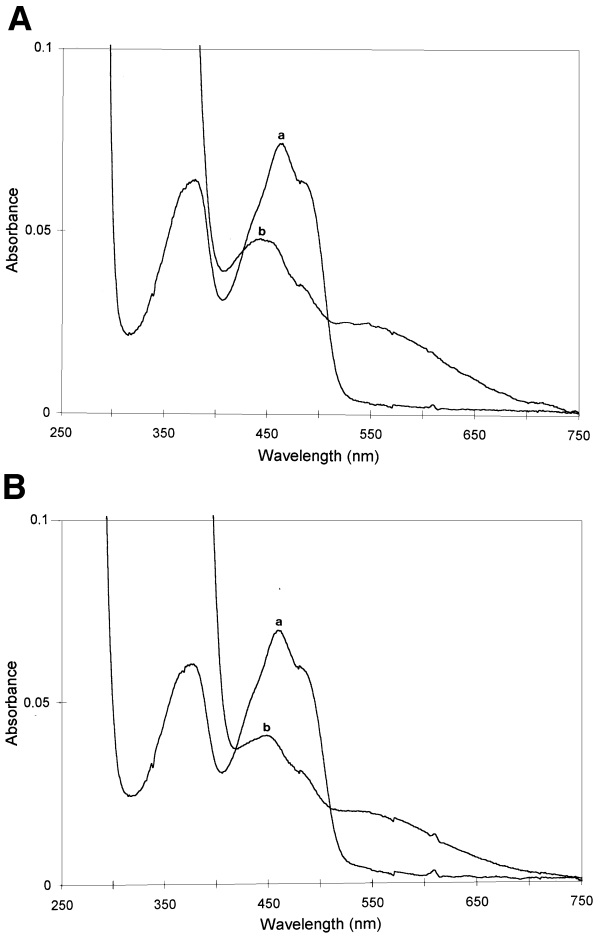

Fig. 1. A: Spectra of wild-type *Pf*TrxR (in 50 mM potassium phosphate buffer pH 7.6 containing 1 mM EDTA). a: Oxidized wild-type *Pf*TrxR (5.9 μM); b: reduced wild-type *Pf*TrxR. Reduction was achieved by addition of 10 equivalents of NADPH/FAD under anaerobic conditions. B: Spectra of *Pf*TrxRC535AC540A (in 50 mM potassium phosphate buffer pH 7.6 containing 1 mM EDTA). a: Oxidized *Pf*TrxRC535AC540A (5.6 μM); b: reduced *Pf*TrxRC535AC540A. Reduction was achieved by addition of 10 equivalents of NADPH/FAD under anaerobic conditions.

deletion mutant. Using the k_{cat}/K_m ratio as a parameter of enzyme efficiency, the double mutant and *Pf*TrxRΔ9 revealed a 12-fold decrease for DTNB reduction, whereas both single mutations affected this ratio by a factor of 4. Remarkably, all mutant proteins were inactive with *E. coli* Trx-S_2 – indicating that both residues are equally important for the reduction of the peptide substrate thioredoxin – whereas the wild-type enzyme reacted with a specific activity of 6 U/mg.

3.3. Spectral analysis of PfTrxRC535AC540A

The absorption spectra of *Pf*TrxRC535AC540A are similar to those of the wild-type protein (Fig. 1). Both proteins have their absorption maximum at 461 nm in the oxidized state and show the characteristic flavin spectrum. Upon reduction with an excess of NADPH, both wild-type enzyme and double mutant *Pf*TrxRC535AC540A formed a flavin thiolate charge transfer complex band at 550 nm and revealed a shift in λ_{max} to 449 nm. The spectra obtained for both proteins are almost congruent implying that the C-terminal cysteine residues of *Pf*TrxR do not interact with the protein bound flavin as previously reported for the redox-active cysteines of *Pf*TrxR [5].

4. Discussion

Mammalian TrxRs possess a penultimate SeCys adjacent to a cysteine residue [4,9,22]. The presence of a SeCys in the proteins and its location suggest a function of this SeCys/Cys pair in the catalysis of the mammalian enzyme. As shown by Arscott et al. [4], this amino acid pair is titratable with dithionite, probably representing an additional third redox-active component within the protein. Further, the location of the additional redox-active residues at the C-terminus of the protein has implications for the accessibility for possible interactions with disulfide substrates during catalysis. These data are reminiscent of mercuric ion reductases, which also contain a carboxy-proximal cysteine pair – albeit no SeCys residue – involved in the reduction of Hg(II) ions [12–14]. In mutant proteins of mercuric ion reductase which lack these Cys residues the DTNB reduction is unimpaired, although the mercuric ion reductase activity is decreased [12].

*Pf*TrxR, another member of the high M_r TrxRs, shows a high degree of similarity to the mammalian enzyme, however, the primary structure at the C-terminus reveals some differences: the parasite amino acid sequence has an extension of five residues (compared with the human TrxR) and the penultimate residue is a normal cysteine rather than a SeCys. The carboxy-proximal cysteine (Cys^{540}) is separated by four amino acids from the second cysteine (Cys^{535}), rather than being located adjacent. It was previously shown that the residues Cys^{88} and Cys^{93} represent the redox-active disulfide and that residue His^{509} – located within the C-terminal region of the protein – is most probably the active site base, in analogy to GR [5]. The knowledge gathered about the importance of the carboxy-terminal cysteine residues for the catalysis of mercuric ion reductases and the potential role of the penultimate SeCys/Cys pair for human TrxR as an additional redox-active component led us to investigate the function of Cys^{540} and

Table 3
Apparent kinetic parameters for recombinant wild-type and mutant *Pf*TrxRs

Construct	Spec. activity (U/mg)	k_{cat} (min^{-1})	K_m DTNB (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
WT	10.41 ± 0.1	624	0.49 ± 0.02	1273
C535A	6.49 ± 0.8	390	1.47 ± 0.27	264
C540A	11.3 ± 0.2	678	1.75 ± 0.26	387
C540AC535A	7.15 ± 0.3	429	4.12 ± 0.60	104
*Pf*TrxRΔ9	6.25 ± 0.7	375	4.07 ± 0.16	92

Data represent means (± S.E.M.) of two different enzyme preparations. The kinetic properties of the recombinant proteins were determined by the DTNB reduction assay. The mixture (1 ml; 20°C) consisted of 100 mM potassium phosphate buffer, pH 7.6, 0.2 mg/ml bovine serum albumin, 2 mM EDTA, 1–3 μg/ml recombinant enzyme, 200 μM NADPH and 0.03–5 mM DTNB. The change in absorbance was monitored spectrophotometrically at 412 nm (Uvikon 932, Kontron). The activity was calculated using the molar absorption coefficient of thionitrobenzoate (TNB) at 412 nm (13 600 M^{-1} cm^{-1} per TNB unit).

Cys^{535} for the catalytic activity of *Pf*TrxR. The C-terminal nine amino acids (Table 1) were deleted, in order to investigate if these residues are functionally involved in catalysis at all. The protein still shows reactivity with DTNB (Table 3), but the reducing activity with *E. coli* Trx-S_2 was eliminated. This significant effect of the C-terminal deletion suggested that the C-terminal thiols may be involved in the reduction of the peptide thioredoxin but are not absolutely necessary for DTNB reduction. By using site-directed mutagenesis we addressed the question whether both cysteine residues are actually involved in this change of reactivity. The characterization of the single and double mutant proteins *Pf*TrxRC535A, *Pf*TrxRC540A and *Pf*TrxRC535AC540A underlines the function of these thiols in the thioredoxin reduction process. All three enzymes are not active with *E. coli* Trx-S_2, although they still show reducing activity with DTNB (Table 3). The specific activities of the mutant proteins with DTNB as a substrate are not as much affected as the K_m values, which are drastically increased. The absence of any detectable reducing activity in all mutant proteins with the peptide substrate *E. coli* Trx-S_2 suggests that both C-terminal cysteines are crucially involved in substrate coordination and/or electron transfer during reduction of the peptide substrate.

To elucidate the relative topology and the possible interaction of the C-terminal cysteines with the protein bound flavin the absorbance spectra of the double mutant were investigated. The spectra of the C-terminal mutants and the wild-type enzyme are remarkably similar, having identical absorbance coefficients and absorbance maxima in both reported redox states (Fig. 1A,B). The absence of perturbation of the absorbance spectra of *Pf*TrxRC535AC540A suggests that the C-terminal cysteines are not in close contact with the isoalloxazine moiety of the protein bound flavin in contrast to the redox-active disulfide (Cys^{88} and Cys^{93}) as previously reported for this protein [5]. Whether these cysteine residues are involved in the reduction of the bulky substrate thioredoxin and/or whether they are responsible for substrate coordination remains to be further investigated.

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