# Thioredoxin Reductase from *Plasmodium falciparum*: Evidence for Interaction between the C-Terminal Cysteine Residues and the Active Site Disulfide−Dithiol†

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ABSTRACT: Thioredoxin reductase (TrxR) catalyzes the reduction of thioredoxin by NADPH. TrxR from *Plasmodium falciparum* (*Pf*TrxR) is a homodimer with a subunit $M_r$ of 59 000. Each monomer contains one FAD and one redox active disulfide. Despite the high degress of similarity between *Pf*TrxR and the human TrxR, their primary structures present a striking difference in the C-terminus. *Pf*TrxR has two cysteine residues near the C-terminal Gly, while the human TrxR contains a Cys-SeCys dipeptide penultimate to the C-terminal Gly. It has been proposed that the C-terminal cysteines (as a cystine) of *Pf*TrxR are involved in catalysis by an intramolecular dithiol−disulfide interchange with the nascent redox active dithiol. To investigate the proposed function of the C-terminal cysteines of *Pf*TrxR, each has been changed to an alanine [Gilberger, T.-M., Bergmann, B., Walter, R. D., and Müller, S. (1998) *FEBS Lett. 425*, 407−410]. The single C-terminal cysteine remaining in each mutant was modified with 5,5′-dithiobis-(2-nitrobenzoic acid) to form mixed disulfides consisting of the enzyme thiol and thionitrobenzoate (TNB). In reductive titrations of these mixed disulfide enzymes, 1 equiv of TNB anion was released upon reduction of the enzyme itself, while control experiments in which mutants without C-terminal cysteine were used showed little TNB anion release. This suggests that each of the C-terminal cysteines as a TNB mixed disulfide does mimic the proposed electron acceptor in the C-terminus. Analysis of the rapid reaction kinetics showed that the C-terminal mixed disulfide of the modified enzyme is reduced at a rate which is comparable with the turnover number of the wild type enzyme.

Thioredoxin reductase (TrxR)[1] catalyzes the reduction of thioredoxin (Trx) by NADPH (*1*, *2*). The substrate Trx is a small monomeric protein that contains one redox active disulfide. Trx in the reduced state is an important cellular disulfide reductant, e.g., in the reduction of ribonucleotide to deoxyribonucleotide catalyzed by ribonucleotide reductase (*3*). TrxRs isolated from a wide variety of prokaryotic and eukaryotic species have been characterized (*2*, *4*−*8*). Two classes of TrxR based on differences in molecular weight, the location of the active site residues, the catalytic mechanism, and the amino acid sequence homology exist (*2*). They are low-molecular weight TrxR from prokaryotes, fungi, and plants having a subunit $M_r$ of 35 000 and high-molecular weight TrxR from higher eukaryotes, including human, bovine, and *Plasmodium falciparum*, with a subunit $M_r$ of 55 000 or 59 000 (*2*, *4*−*9*). TrxRs of both classes are members of the pyridine nucleotide disulfide oxidoreductase family of flavoenzymes, including glutathione reductase, lipoamide dehydrogenase, and mercuric reductase (*1*, *2*, *10*). This assignment is based on similarities such as the substrate specificity (pyridine nucleotides and dithiol−disulfide compounds), the presence of a redox active disulfide interacting directly with the FAD, the homodimeric structures, and amino acid sequence homology (*1*, *2*). The three-dimensional structures and the mechanistic studies show that glutathione reductase, lipoamide dehydrogenase, and mercuric reductase, as well as high-molecular weight TrxR, are very closely related (*1*, *2*, *11*, *12*), whereas the low-molecular weight enzyme, *Escherichia coli* TrxR, which shows distinct mechanism and structural characteristics, is only distantly related to the other members (*13*, *14*).

*P. falciparum* is a causative agent of malaria. TrxR from *P. falciparum* (*Pf*TrxR) has been cloned and expressed in *E. coli* (*15*, *16*) and characterized (*9*). The deduced amino acid sequence of *Pf*TrxR is 44% identical with that of human TrxR, 35% identical with that of human glutathione reductase, whereas only 25% identical with that of *E. coli* TrxR

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[1] Abbreviations: TrxR, thioredoxin reductase; Trx, thioredoxin; *Pf*TrxR, TrxR from *Plasmodium falciparum*; C535A, altered form of *Pf*TrxR in which Cys535 has been changed to alanine; C540−TNB, C535A reacted with DTNB; C540A, altered form of *Pf*TrxR in which Cys540 has been changed to alanine; C535−TNB, C540A reacted with DTNB; C535A/C540A, altered form of *Pf*TrxR in which both Cys535 and Cys540 have been changed to alanine; Mut-9, altered form of *Pf*TrxR with nine C-terminal amino acid residues deleted; $E_{ox}$, enzyme with the flavin and redox active disulfide in the oxidized state; $EH_2$, two-electron-reduced enzyme in which the redox active dithiol forms a charge transfer complex with FAD; $EH_4$, four-electron-reduced enzyme with reduced flavin and the active site dithiol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB anion, 5-thio-2-nitrobenzoate anion; DTT, dithiothreitol.

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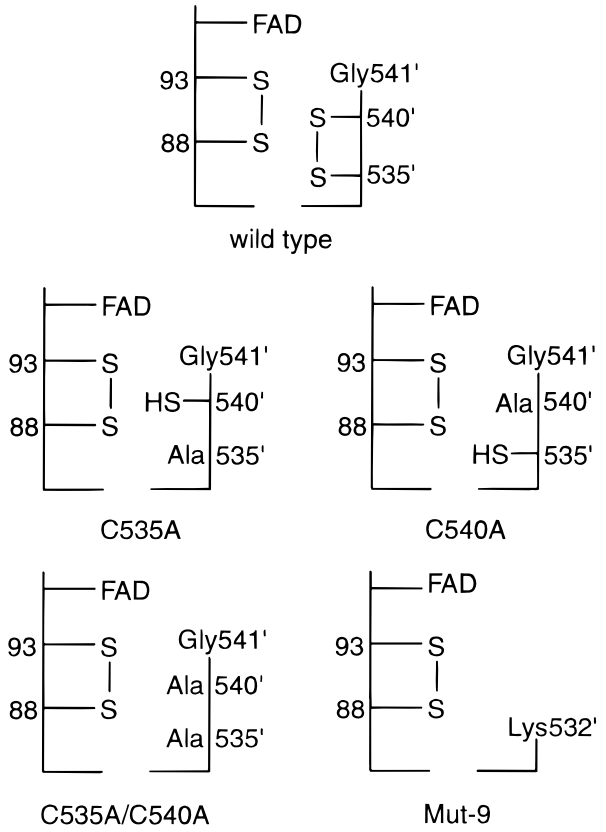
Scheme 1: Representation of Wild Type *Pf*TrxR and Forms of the Enzyme Altered by Site-Directed Mutagenesis




(*15*). It was determined that the redox active disulfide of *Pf*TrxR is made up of Cys88 and Cys93 using site-directed mutagenesis (*9*). The sequence around these active site cysteines is highly conserved in the high-$M_r$ TrxR, including *Pf*TrxR and human TrxR. One major difference between *Pf*TrxR and human TrxR is found in the C-terminal region. Human TrxR contains a cysteine-selenocysteine (Cys-SeCys-Gly) at the C-terminus (*8*). Reductive titration of human TrxR indicates that the C-terminal Cys-SeCys is in redox communication with the active site disulfide−dithiol (*2*). In contrast, *Pf*TrxR has two cysteine residues (Cys535 and Cys540) in a C-terminal sequence, Cys-x-x-x-x-Cys-Gly. Mutants derived by site-directed mutagenesis suggest that the C-terminal cysteines of *Pf*TrxR may be involved in communication with the redox active disulfide−dithiol (*17*). It should be noted that a similar intramolecular dithiol−disulfide interchange is also present in mercuric reductase, which is in the same enzyme family with TrxR and contains two C-terminal cysteines (*18*). The clear chemical difference between the Cys-SeCys in human thioredoxin reductase and the Cys-x-x-x-x-Cys in thioredoxin reductase from *P. falciparum* should stimulate rational drug design.

To understand the function of the C-terminal cysteine residues, four alterations of *Pf*TrxR have been effected (*17*). One or both of the C-terminal cysteines have been changed to alanine(s), resulting in the mutants C535A and C540A and the double mutant C535A/C540A. Mut-9 has nine amino acid residues at the C-terminus deleted, including both Cys535 and Cys540 (*17*) (Scheme 1). C535A and C540A have one remaining C-terminal cysteine. In this work, we have studied the four modified forms of *Pf*TrxR by comparing them with the wild type enzyme. Evidence from the reductive titrations and pre-steady-state kinetics is presented to support the proposition that the C-terminal cysteines are in redox communication with the active site disulfide.

## MATERIALS AND METHODS

*Reagents.* NADPH, sodium dithionite, and 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol was purchased from Aldrich Chemical Co. (Milwaukee, WI). The wild type enzyme and the *Pf*TrxR mutants C535A, C540A, C535A/C540A, and Mut-9 were prepared as described previously (*9*, *17*). All other reagents and buffer salts were of the highest quality available.

All experiments were conducted in 50 mM potassium phosphate buffer containing 1 mM EDTA at pH 7.6, unless otherwise indicated. The concentrations of all the mutants and wild type *Pf*TrxR were calculated from the absorbance at 460 nm using an extinction coefficient of 11 300 $M^{-1}$ $cm^{-1}$.

*Anaerobiosis.* All reductive titrations were performed under anaerobic conditions at 25 °C. Anaerobiosis was achieved by alternately degassing under vacuum and equilibration with ultrapure nitrogen (99.999%) for eight cycles as described previously (*19*, *20*). Enzyme solutions were protected from light during anaerobiosis and reductive titrations.

NADPH solutions were prepared in 2 mM Tris base solution (pH 9.0) and were made anaerobic by 10 alternating cycles of vacuum and nitrogen equilibration with vortexing (valve between sample and pump closed when sample was under vacuum). The concentration of anaerobic NADPH solution was determined by measuring the absorbance at 340 nm of diluted samples using an extinction coefficient of 6220 $M^{-1}$ $cm^{-1}$.

*Sodium Dithionite Titrations.* Titrations of C535A and C540A with sodium dithionite were performed using the method described previously (*21*) with minor modifications. Anaerobic sodium dithionite solution was prepared in 50 mM pyrophosphate buffer at pH 9.0 and quantitated by anaerobic titration of lumiflavin-3-acetic acid. The enzyme samples for dithionite titration contain 2 or 10% methyl viologen, which is used as an electron mediator between dithionite and the enzyme.

Prior to dithionite titration, the C535A or C540A (about 20 $\mu$M) enzyme was treated with a 100-fold excess of DTT to make sure that the remaining C-terminal cysteine residue was in the reduced state. Upon reaction with DTT, the enzyme turned to a reddish brown color, indicating reduction of the redox active disulfide and formation of a thiolate−FAD charge transfer complex. The DTT-treated enzyme was dialyzed for 12 h to remove excess DTT. The active site dithiol was reoxidized to disulfide during the dialysis (as was shown by spectral analysis), but the C-terminal cysteine remained in the reduced state as shown in the subsequent DTNB modification.

In a typical dithionite titration, a solution of 15−20 nmol of C535A or C540A enzyme was placed in a cuvette with two sidearms similar to that described previously (*20*), and the environment was made anaerobic. While the cuvette was connected to the nitrogen line, it was opened and a steady stream of nitrogen was allowed to flow out. An anaerobic

dithionite solution (100 $\mu$L at 2 mM) was placed in each sidearm; the cuvette was closed, and four additional cycles of vacuum and nitrogen equilibration were applied. The dithionite in the sidearms assisted in maintaining anaerobiosis. The enzyme was reduced anaerobically by sodium dithionite added incrementally from a gastight Hamilton syringe, and a spectrum was recorded after each addition of dithionite using a Perkin-Elmer spectrophotometer (*20*).

*DTNB Modifications.* Prior to DTNB modification, enzyme samples were treated with DTT and dialyzed as described above. The stock solution of DTNB (25 mM) was made in 0.1 M sodium acetate buffer at pH 5.0. A solution of 20 $\mu$M C535A, C540A, C535A/C540A, Mut-9, or wild type enzyme was incubated with a 100-fold excess of DTNB in 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA at room temperature for 60 min. The mixture was dialyzed at 4 °C for 12 h to remove excess DTNB and the released TNB anion.

The amount of the TNB−enzyme mixed disulfide formed was quantified as follows. About 5 $\mu$M modified protein was incubated with 2 mM DTT for 1 h at room temperature. Released TNB anion was separated from the enzyme using an Amicon Centricon Concentrator unit (Beverly, MA) and quantified from the absorbance at 412 nm using an extinction coefficient of 13 600 $M^{-1}$ $cm^{-1}$.

*NADPH Titrations.* The modified proteins were titrated with NADPH anaerobically in 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA and 0.5 M sodium chloride. When the titration was performed in the same buffer without 0.5 M sodium chloride, turbidity was observed after reduction of the modified enzymes by NADPH. After NADPH titration, the reduced enzyme was reoxidized by opening the cuvette to air. The released free TNB anion was separated from the enzyme using an Amicon Centricon Concentrator unit and quantified from the absorbance at 412 nm.

NADPH titration of *Pf*TrxR C540A was performed in 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA without sodium chloride; turbidity was not observed in this case. The effect of $NADP^+$ on the thiolate−FAD charge transfer complex was examined by repeating the NADPH titration of C540A in the presence of 30 equiv of $NADP^+$.

*Rapid Reaction Spectrophotometry*. The rapid reaction spectrophotometry and the data analysis of the pre-steady-state kinetics were performed as described previously (*22*, *23*). The enzyme solution (13.7 $\mu$M C535A or 16.9 $\mu$M C540−TNB) in 50 mM potassium phosphate (pH 7.6) and 1 mM EDTA was made anaerobic in a tonometer, and then it was attached to the instrument. The solution of NADPH in the same buffer was bubbled with nitrogen for 10 min prior to attachment. The two solutions were mixed rapidly at 4 °C, and kinetic traces at 460 and 540 nm (photomultiplier detector) and spectra (diode array detector) were collected. In the case of C540−TNB, both the enzyme and NADPH solutions contained 0.5 M NaCl to prevent protein precipitation upon reduction of the mixed disulfide.

## RESULTS

*Reductive Titrations of PfTrxR C535A and C540A.* Due to the extensive sequence homology among high-molecular weight TrxR, lipoamide dehydrogenase, and glutathione reductase, it is expected that the redox properties of *Pf*TrxR will be similar to those of human TrxR and glutathione reductase, and different from those of *E. coli* TrxR (*2*). In the reductive titrations of glutathione reductase and human TrxR, a distinct $EH_2$ species is observed as a stable intermediate that exhibits a long-wavelength band centered around 540 nm due to the formation of a thiolate−FAD charge transfer complex (*2*, *22*, *24*).

Dithionite titration of *Pf*TrxR C540A resembles that of human TrxR and glutathione reductase. As shown in Figure 1, the titration proceeds in two phases. An absorbance increase at 540 nm and a slight decrease at 460 nm were observed in the first phase. The spectrum at the end of the first phase has a long-wavelength band centered around 540 nm which represents a thiolate−FAD charge transfer complex (Figure 1A). After correction for the oxygen present at the beginning, 1 equiv of dithionite was used in this phase (Figure 1B, inset). A set of four isosbestic points (at 507, 443, 397, and 360 nm) indicates that only two species ($E_{ox}$ and $EH_2$) are involved in the first phase. The last spectrum (g, 1.3 equiv of dithionite) in the first phase shows an increase in absorbance at 540 nm, but does not fit the isosbestic points, indicating the beginning of FAD reduction in the transition from the first to the second phase. In the second phase, decreases of absorbance at both 460 and 540 nm were observed, indicating the reduction of the thiolate−FAD charge transfer complex to $EH_4$, in which both the redox active disulfide and the flavin have been reduced. The final spectrum (n) after addition of 2.7 equiv of dithionite shows increased absorbance at 315, 395, and 610 nm, indicative of methyl viologen radical and excess dithionite, as expected when the enzyme is fully reduced (Figure 1B). One equivalent of dithionite was used in the second phase. The final species ($EH_4$) was stable; under anaerobic conditions, there was no reoxidation of the $EH_4$ species over the course of 14 h. Dithionite titration of C535A gave an almost identical result (data not shown).

NADPH can only reduce wild type *Pf*TrxR, *Pf*TrxR C540A, and *Pf*TrxR C535A to the $EH_2$ species, in contrast to the reduction with dithionite (Figure 2). The final spectrum in the NADPH titration of C540A exhibits characteristics that are different from those of the $EH_2$ species observed in the dithionite titration. The main flavin band is somewhat more reduced in the presence of excess NADPH; the $\epsilon_{450nm}$ of the $EH_2$ species is 8900 $M^{-1}$ $cm^{-1}$ in the dithionite titration and 6700 $M^{-1}$ $cm^{-1}$ in the NADPH titration, and the shape of the thiolate−FAD charge transfer band is different, extending beyond 750 nm in the NADPH reduction (cf. Figures 1A and 2). It has been proposed that these differences exist because of the binding of $NADP^+$ and/or NADPH to the two-electron-reduced species (*24*). The spectra of C540A display the characteristics observed in similar titrations of glutathione reductase and human TrxR. Briefly, NADPH enhanced the thiolate−FAD charge transfer band (cf. spectra c and d in Figure 2), and $NADP^+$ caused a shift to longer wavelengths (lower energy, Figure 2, inset).

*DTNB Modification.* The mutant forms of *Pf*TrxR, C535A or C540A, were reacted with DTNB to determine whether they are in redox contact with active site nascent dithiol. During the reaction, the single remaining C-terminal cysteine was modified, resulting in a mixed disulfide between the

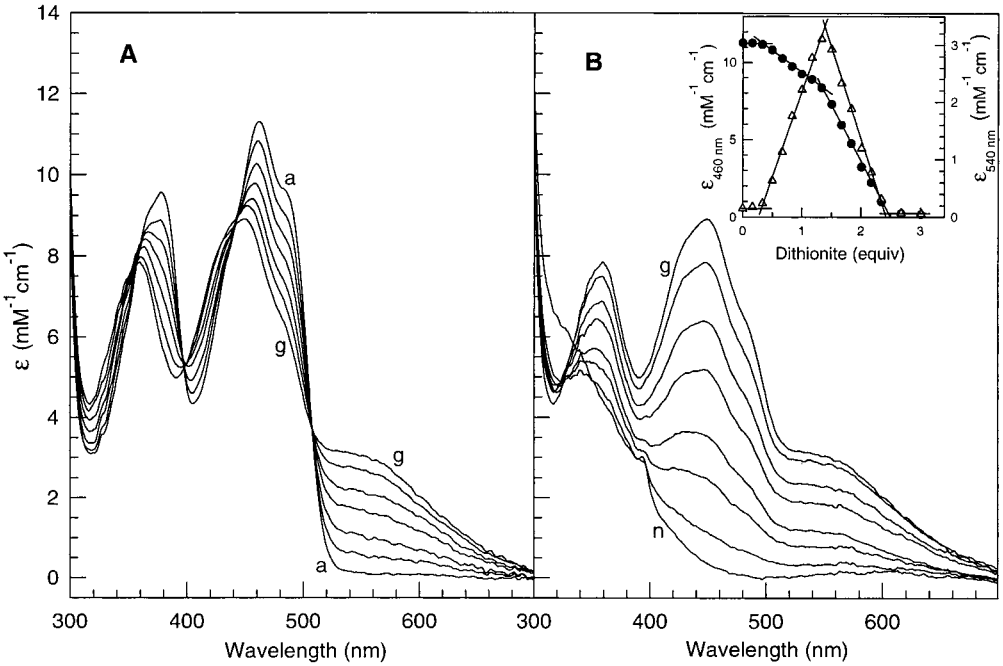



FIGURE 1: Titration of *Pf*TrxR C540A with sodium dithionite. A solution of 22.1 nmol of C540A in 1.0 mL of 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA and 0.44 nmol of methyl viologen was titrated anaerobically with 1.85 mM sodium dithionite at 25 °C. (A) The first phase of the titration. The spectra were recorded for the mixture after addition of 0, 0.50, 0.67, 0.84, 1.00, 1.17, and 1.34 equiv of sodium dithionite (a−g, respectively). (B) The second phase of the titration. The spectra were recorded for the mixture after addition of 1.34, 1.51, 1.67, 1.84, 2.01, 2.18, 2.34, and 2.68 equiv of sodium dithionite (g−n, respectively). In the inset are plots of $\epsilon_{460nm}$ (●) and $\epsilon_{540nm}$ (△) vs the number of equivalents of sodium dithionite.

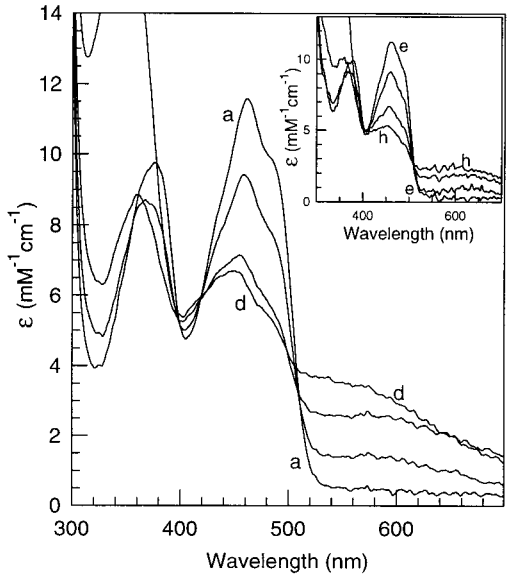



FIGURE 2: Titration of *Pf*TrxR C540A with NADPH. A solution of 21.8 nmol of C540A in 1.0 mL of 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA was titrated anaerobically with 1.73 mM NADPH at 25 °C. The spectra were recorded for the mixture after addition of 0, 0.48, 1.11, and 3.17 equiv of NADPH (a−d, respectively). (Inset) A solution of 14.1 nmol of C540A in 1.0 mL of 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA and 422 $\mu$M $NADP^+$ was titrated anaerobically with 1.68 mM NADPH at 25 °C. The spectra were recorded for the mixture after addition of 0, 0.48, 1.19, and 3.11 equiv of NADPH (e−h, respectively).

enzyme and TNB (C535−TNB or C540−TNB) as described in Scheme 1 and by the following reaction, where Φ-S-S-Φ represents DTNB.

$$\text{Enz-S}^- + \Phi\text{-S-S-}\Phi \rightleftharpoons \text{Enz-S-S-}\Phi + \Phi\text{-S}^-$$

Table 1: Quantification of the Amount of TNB Released from the Enzymes Reacted with DTNB upon Treatment with DTT

| enzyme | TNB anion released from the DTT treatment of the modified enzymes (equiv) |
|---|---|
| C535A | 1.88 |
| C540A | 2.02 |
| wild type | not determined |
| C535A/C540A | 0.84 |
| Mut-9 | 1.00 |

Release of the TNB anion makes the proposed intramolecular dithiol−disulfide interchange reaction readily detectable. If the C-terminal cysteines are able to accept reducing equivalents passed from the active site dithiol, then the mixed disulfide C535−TNB or C540−TNB would be reduced during a reductive titration, releasing a TNB anion. The release of TNB anion would be observed as an absorbance decrease at 340 nm due to the breakdown of the mixed disulfide and an absorbance increase at 412 nm due to the formation of free TNB anion (see below; Figure 3A).

The reactions of DTNB with the altered forms of *Pf*TrxR were followed by monitoring the release of the TNB anion at 412 nm. The reaction of C535A and C540A with DTNB results in a rapid increase of $A_{412nm}$. The reaction was biphasic (see below). The modified enzymes exhibit spectra with significantly enhanced $A_{340nm}$ values, due to the formation of the mixed disulfide (*25*). The modifications to each enzyme were quantified by removing the TNB anion by treatment of the modified enzyme with DTT. As shown in Table 1, it is clear that the mutants with one remaining C-terminal cysteine (C535A and C540A) have about 2 equiv of TNB, but the mutants without C-terminal cysteine (C535A/C540A and Mut-9) have only 1 equiv. *Pf*TrxR possesses 13 cysteine residues, two in the active site, two at

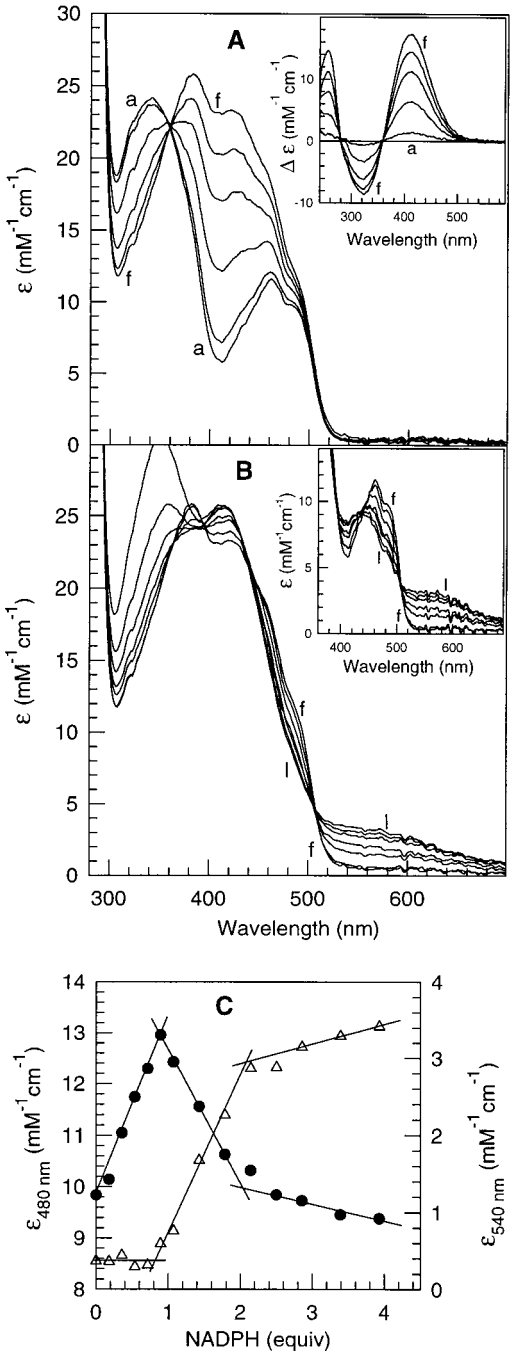



FIGURE 3: Titration of C540−TNB (DTNB-modified *Pf*TrxR C535A) with NADPH. A solution of 17.1 nmol of C540−TNB in 0.95 mL of 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA and 0.5 M NaCl was titrated anaerobically with 1.53 mM NADPH at 25 °C. (A) The first phase of the titration. The spectra were recorded for the mixture after addition of 0, 0.18, 0.36, 0.54, 0.71, and 0.89 equiv of NADPH (a−f, respectively). (Inset) The difference spectra derived from subtraction of the starting spectrum from each spectrum in the first phase. (B) The second phase of the titration. The spectra were recorded for the mixture after addition of 0.89, 1.07, 1.43, 1.79, 2.14, 2.86, and 3.93 equiv of NADPH (f−l, respectively). (Inset) The difference spectra derived by subtraction of the absorbance change that occurred in the first phase from each spectrum in the second phase. (C) The plots of $\epsilon_{480nm}$ (●) and $\epsilon_{540nm}$ (△) vs the number of equivalents of NADPH.

the C-terminus, and the remainder buried elsewhere in the structure. The data indicate that one or more of the latter can be partially modified by DTNB (biphasic reaction).

Table 2: Quantification of the Amount of TNB Anion Released in the Reductive Titrations of the Enzymes Modified by Treatment with DTNB

| enzyme | TNB anion released before the reduction of the active site disulfide (equiv)[a] | TNB anion released in the entire titration (equiv) |
|---|---|---|
| C540−TNB | 1.26 | 1.34 |
| C535−TNB | 0.83 | 1.12 |
| wild type | >0.19 | 0.99 |
| C535A/C540A | 0.07 | 0.41 |
| Mut-9 | 0.04 | 0.24 |

[a] The equivalents of TNB anion released before the reduction of the active site disulfide were calculated from the absorbance increase at 412 nm.

*NADPH Titrations of the Modified Enzymes.* C540−TNB is reduced by NADPH in three phases (Figure 3). Reaction of the modified enzyme with the first 0.9 equiv of NADPH constitutes the first phase, in which the 340 nm band is diminished and the absorbance increases in the region from 360 to 500 nm (Figure 3A,C). The difference spectra (inset of Figure 3A) indicate the formation of a band centered at 412 nm that is identical to the spectrum of free TNB anion and a negative band centered at 320 nm that is caused by the breakdown of the mixed disulfide. Two isosbestic points are observed at 280 and 359 nm, indicating that only two species are present in the first phase, enzyme before and after release of the TNB anion. There is no formation of a long-wavelength band or reduction of the flavin peak. The amount of TNB anion released is 1.26 equiv during the first phase (Table 2). Figure 3B shows the second phase, from 0.9 to 2.1 equiv of NADPH, in which a long-wavelength band was formed which resembles that observed in the NADPH titration of C540A. The absorbance of the main flavin band was decreased as indicated by the flavin shoulder near 480−490 nm. A small further increase of 412 nm was observed in the second phase. The changes at 460 nm in the main flavin band are obscured by the TNB anion, but a correction can be made. After subtraction of the absorbance from the free TNB anion released in the first phase, the difference spectra in the second phase are consistent with normal enzyme reduction (inset of Figure 3B). Spectra from the third phase indicate the expected enhancement of the thiolate−FAD charge transfer complex by NADPH (Figure 3C). Quantitation of the amount of TNB anion released relative to that of the enzyme-bound FAD is given in Table 2. NADPH titration of C535−TNB gives very similar results (Table 2).

The reductive titrations of DTNB-treated C535A/C540A and Mut-9 were similar to one another having only two phases: formation and enhancement of the thiolate−FAD charge transfer complex. These altered forms serve as controls for the titration of C535−TNB and C540−TNB. As shown in Figure 4 for the reduction of Mut-9−TNB, most of the changes in the flavin long-wavelength bands were complete in the first phase. The second phase resembles the third phase in the reduction of C540−TNB. The results are summarized in Table 2.

Wild type *Pf*TrxR, pretreated with DTT and treated with DTNB, behaves in a manner different from that of the other DTNB-treated enzymes in the reductive titration. The first addition of NADPH (0.3 equiv) gives an increase of $A_{412nm}$ corresponding to 0.19 equiv of TNB anion release without

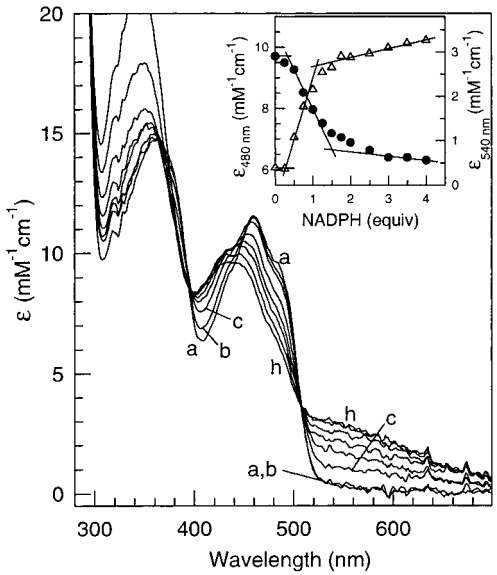



FIGURE 4: Titration of the DTNB-modified *Pf*TrxR Mut-9 with NADPH. A solution of 12.7 nmol of modified Mut-9 in 1.0 mL of 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA and 0.5 M NaCl was titrated anaerobically with 1.59 mM NADPH at 25 °C. The spectra were recorded for the mixture after addition of 0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.75, and 2.50 equiv of NADPH (a−h, respectively). In the inset are shown plots of $\epsilon_{480nm}$ (●) and $\epsilon_{540nm}$ (△) vs the number of equivalents of NADPH.

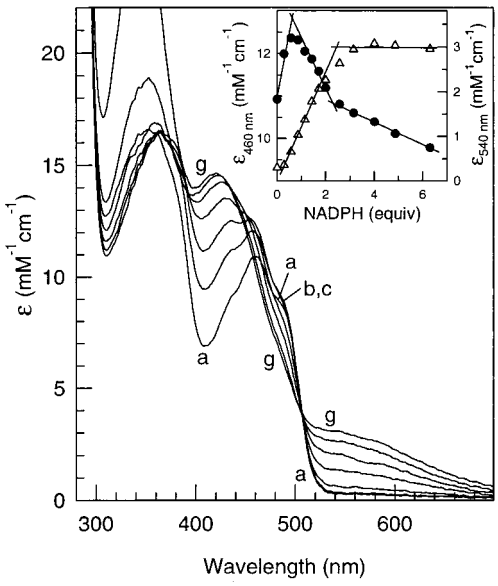



FIGURE 5: Titration of the DTNB-modified wild type *Pf*TrxR with NADPH. A solution of 7.6 nmol of DTNB-modified wild type enzyme in 0.95 mL of 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA and 0.5 M NaCl was titrated anaerobically with 1.09 mM NADPH at 25 °C. The spectra were recorded for the mixture after addition of 0, 0.29, 0.57, 1.15, 1.72, 2.58, and 4.01 equiv of NADPH (a−g, respectively). In the inset are shown plots of $\epsilon_{460nm}$ (●) and $\epsilon_{540nm}$ (△) vs the number of equivalents of NADPH.

loss of the flavin band (as seen in the 485 nm shoulder) or formation of the long-wavelength band (Figure 5). Subsequent additions of NADPH result in the formation of the long-wavelength band and flavin reduction accompanying a further increase of $A_{412nm}$. The total amount of released free TNB anion in this titration was measured to be 1.0 equiv (Table 2). It is possible that the lower than expected yield of TNB anion (1 equiv rather than 2) is due to the partial reoxidation of the C-terminal dithiol by DTNB. This is usually only observed when DTNB is limiting; formation of the first mixed disulfide is followed by formation of the disulfide rather than by reaction with a second molecule of DTNB.

*Pre-Steady-State Kinetics*. Reduction of C535A and C540−TNB has been studied using a rapid reaction spectrophotometer. Figure 6 shows the spectra collected during the reduction of C535A by 1 equiv of NADPH, and the inset shows the continuous absorbance change at 460 and 540 nm. The observed rate constants obtained from the kinetic traces are listed in Table 3. The flavin of C535A was reduced quickly after mixing with NADPH (in the first 20 ms), resulting in a species having a broad long-wavelength band centered around 650 nm. The spectral characteristics of this species, represented by the spectrum at 7.7 ms (dotted), indicate reduction of the flavin and formation of a reduced flavin−$NADP^+$ charge transfer complex, by analogy with thioredoxin reductase from human placenta and glutathione reductase (*2*, *22*). From 20 to 100 ms, an absorbance increase at 460 nm and enhancement of the long-wavelength band were observed. The significant increase of $A_{540nm}$ and little signal change beyond 650 nm in the second phase result in a different shape of the long-wavelength band centered around 550 nm which represents the thiolate−FAD charge transfer complex. An additional phase was observed as a very small signal change at both 460 and 540 nm, but only in the reaction of C535A with 1 equiv of NADPH; in the reduction of C535A by higher concentrations of NADPH, just the first two phases are observed. The dependence of the rates on the NADPH concentration is given in Table 3.

The reaction of C540−TNB with 1 equiv of NADPH proceeds in four phases (Figure 7, inset). The first phase is observed as an absorbance decrease at 460 nm accompanied by the formation of a long-wavelength band. The second phase of the reaction appears as an increase of $A_{460nm}$ and enhancement of the long-wavelength band around 550 nm, which is complete in about 110 ms. As in the reductive half-reaction of C535A, the spectral changes occurring in the first two phases of the reduction of C540−TNB by NADPH are consistent with the reduction of the FAD resulting in a reduced flavin−$NADP^+$ charge transfer complex followed by transfer of the reducing equivalent to the active site disulfide with formation of a thiolate−FAD charge transfer complex.

Two additional phases were observed in the reduction of C540−TNB after formation of the thiolate−FAD charge transfer complex. The third phase involves a significant absorbance increase at 412 nm and little signal change of the long-wavelength band. The increase of $A_{412nm}$ indicates release of free TNB anion due to breakdown of the mixed disulfide between the enzyme and TNB at a rate of 2 $s^{-1}$. The lag at 540 nm suggests that the thiolate−FAD charge transfer complex is being formed and broken down in equal amounts during the reduction of the mixed disulfide (see the Discussion). The last phase was observed as a slight decrease of $A_{540nm}$ and a further increase of $A_{460nm}$ at a very low observed rate constant (0.32 $s^{-1}$) and is not complete at the end of the data collection (10 s).

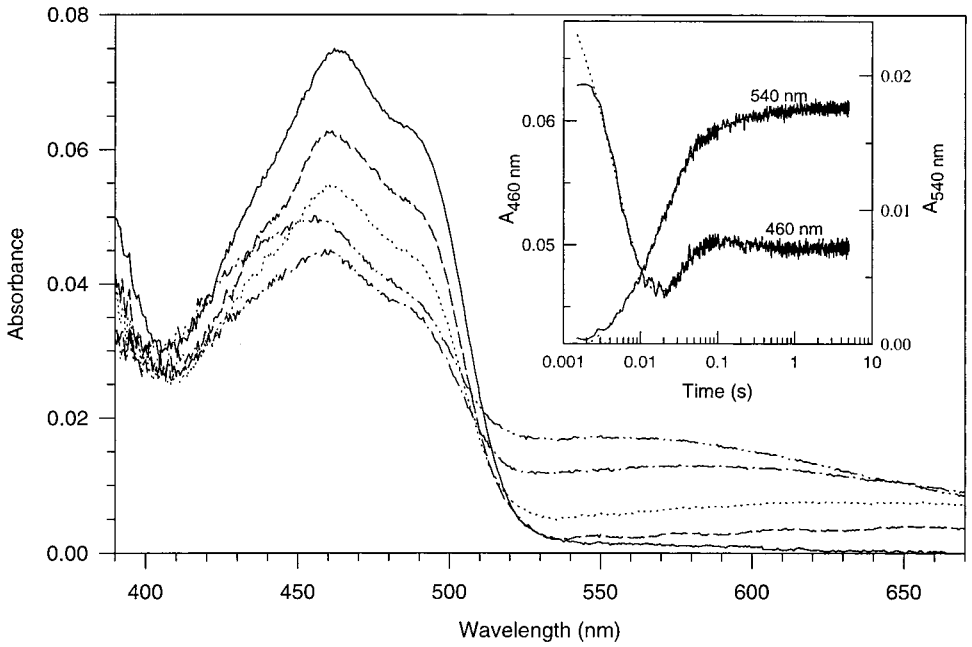



FIGURE 6: Rapid reaction of C535A with NADPH. C535A (6.9 $\mu$M after mixing) was reduced by 1 equiv of NADPH in 50 mM potassium phosphate buffer containing 1 mM EDTA at pH 7.6 and 4 °C. The spectra were recorded for the oxidized enzyme (—), after a 2.3 ms dead time (- − -), and at 7.7 ms (•••), 24 ms (•-•), and 89 ms (••-••). In the inset are shown single-wavelength kinetic traces observed at 460 and 540 nm. The dotted lines show the fits of the observed traces to a sum of three exponentials (460 nm) and two exponentials (540 nm).

Table 3: Summary of the Rate Constants Observed in the Reduction of C535A and C540−TNB by NADPH

| enzyme | $k_{FR}$ ($s^{-1}$)[a,b] | $k_{CT}$ ($s^{-1}$)[a,c] | $k_{IC1}$ ($s^{-1}$)[a] | $k_{IC2}$ ($s^{-1}$)[a] |
|---|---|---|---|---|
| C535A | 190[d] | 47 | – | – |
| C540−TNB | 47[e] | 15 | 2.4 | 0.32 |

[a] $k_{FR}$ is the rate of flavin reduction, and $k_{CT}$ is the rate of formation of the thiolate−FAD charge transfer complex. $k_{IC1}$ and $k_{IC2}$ refer to the first and second step of the intramolecular thiol−disulfide interchange, respectively (see Scheme 2). [b] The data for $k_{FR}$ were observed at 460 nm. [c] The data were averaged from observations at 460 and 540 nm for C535A, and the data were observed at 540 nm for C540−TNB. [d] Rate with 1 equiv of NADPH; $k_{FR}$ gave a $V_{max}$ of 665 ± 35 $s^{-1}$ and a $K_d$ of 16.5 ± 3.1 $\mu$M. [e] Rate with 1 equiv of NADPH; $k_{FR}$ gave a $V_{max}$ of 578 ± 80 $s^{-1}$ and a $K_d$ of 184 ± 59 $\mu$M.

## DISCUSSION

*Pf*TrxR, as a high-molecular weight thioredoxin reductase, is expected to exhibit redox properties similar to those of human TrxR and the other members of this enzyme family such as glutathione reductase and lipoamide dehydrogenase, but distinct from those of *E. coli* TrxR (*2*). The midpoint potentials of $E_{ox}$−$EH_2$ and $EH_2$−$EH_4$ couples are separated by more than 50 mV in lipoamide dehydrogenase and glutathione reductase (*26*), while the separation of the midpoint potentials between FAD−$FADH_2$ and disulfide−dithiol couples is only 11 mV in the case of *E. coli* TrxR (*27*). As a consequence in the reductive titrations of lipoamide dehydrogenase and glutathione reductase, a distinct $EH_2$ species is observed as a stable intermediate which exhibits a long-wavelength absorbance centered around 540 nm due to a thiolate−FAD charge transfer complex (*28*, *29*). A stable $EH_2$ species is not observed in the reductive titrations of *E. coli* TrxR (*5*). It has been reported previously that the wild type *Pf*TrxR forms a thiolate−FAD charge transfer complex upon reduction by NADPH (*9*), and a similar $EH_2$ species was also observed in the reductive titration of human TrxR (*2*). It is our hypothesis that the C-terminal cysteines are involved in the catalysis of *Pf*TrxR, based on the fact that human TrxR and bacterial mercuric reductase have similar intramolecular interactions between the redox active dithiol and the C-terminal Cys-SeCys or Cys-Cys, respectively (*2*, *18*). The involvement of the C-terminal cysteines in catalysis should not be possible in C535A and C540A, since one of the C-terminal cysteines has been changed to alanine, and the single remaining C-terminal cysteine would not be expected to interact with the active site dithiol. Therefore, the static dithionite titrations of mutants C535A and C540A will give a simplified pattern (two phases, reduction of the active center disulfide followed by flavin reduction) compared with that of the wild type enzyme (three phases, reduction of the C-terminal disulfide, reduction of the active center disulfide, and flavin reduction) (Scheme 1).[2] Of course, reducing equivalents reach the C-terminal disulfide via the FAD and the active center disulfide.

The mutants are reduced stoichiometrically by dithionite, the first equivalent of dithionite (two electrons) forming the thiolate−FAD charge transfer complex ($EH_2$ species) and the second equivalent reducing $EH_2$ to $EH_4$ in which both the redox active disulfide and the flavin are in the reduced state (Figure 1). On the basis of these results, it is clear that the active site disulfide and the flavin are the only reducible groups in C535A and C540A, and there is no additional electron acceptor in communication with the active site (Scheme 1). In contrast, wild type *Pf*TrxR requires 2 equiv of dithionite to form the thiolate−FAD charge transfer complex, one for each disulfide, and an additional 1 equiv to reduce the flavin.[2] Titrations with NADPH have also shown (Figure 2) that *Pf*TrxR is similar to human TrxR in its behavior (*2*). Two well-characterized intermediates were observed in the two-electron-reduced enzyme, the reduced

[2] L. D. Arscott and C. H. Williams, Jr., unpublished results.

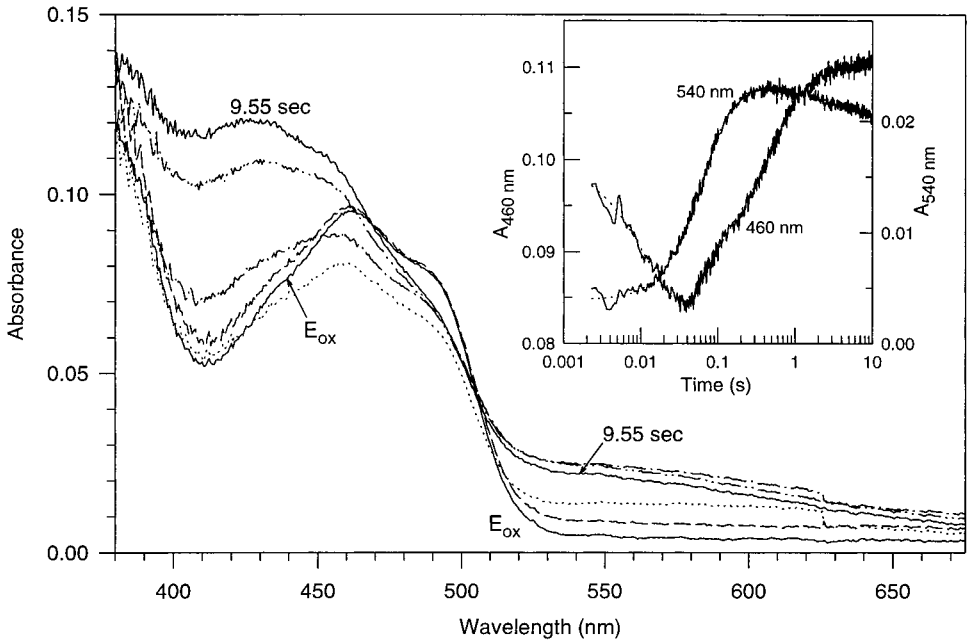



FIGURE 7: Rapid reaction of C540−TNB (DTNB-modified C535A) with NADPH. C540−TNB (8.5 $\mu$M after mixing) was reduced by 1 equiv of NADPH in 50 mM potassium phosphate buffer containing 1 mM EDTA and 0.5 M sodium chloride at pH 7.6 and 4 °C. The spectra were recorded for the oxidized enzyme (—), after a 2.3 ms dead time (- − -), and at 7.7 ms (•••), 24 ms (•-•), 89 ms (••-••), and 9.55 s (the solid line with the highest $A_{412nm}$). In the inset are shown single-wavelength kinetic traces observed at 460 and 540 nm. The dotted lines show the fits of the observed traces to a sum of four exponentials (460 nm) and three exponentials (540 nm).

flavin−$NADP^+$ charge transfer complex and the thiolate−FAD charge transfer complex (*1*).

To demonstrate the proposed intramolecular communication between the C-terminal disulfide and the redox active, nascent dithiol, C535A and C540A have been modified by reaction with DTNB to yield the mixed disulfide between the remaining enzyme thiol and TNB. In a reductive titration of the modified enzyme, the reaction of the mixed disulfide can be monitored easily by observing the absorbance change at 340 nm where the mixed disulfide absorbs and at 412 nm where the TNB anion absorbs. This study has shown that C535−TNB and C540−TNB incorporate approximately 2 equiv of TNB, while only 1 equiv of TNB is present in the DTNB-treated C535A/C540A and Mut-9 that serve as controls (Table 1). The difference between the two groups implies that 1 of the 2 equiv of TNB comes from the mixed disulfide with the single remaining cysteine at the C-terminus (Scheme 1).

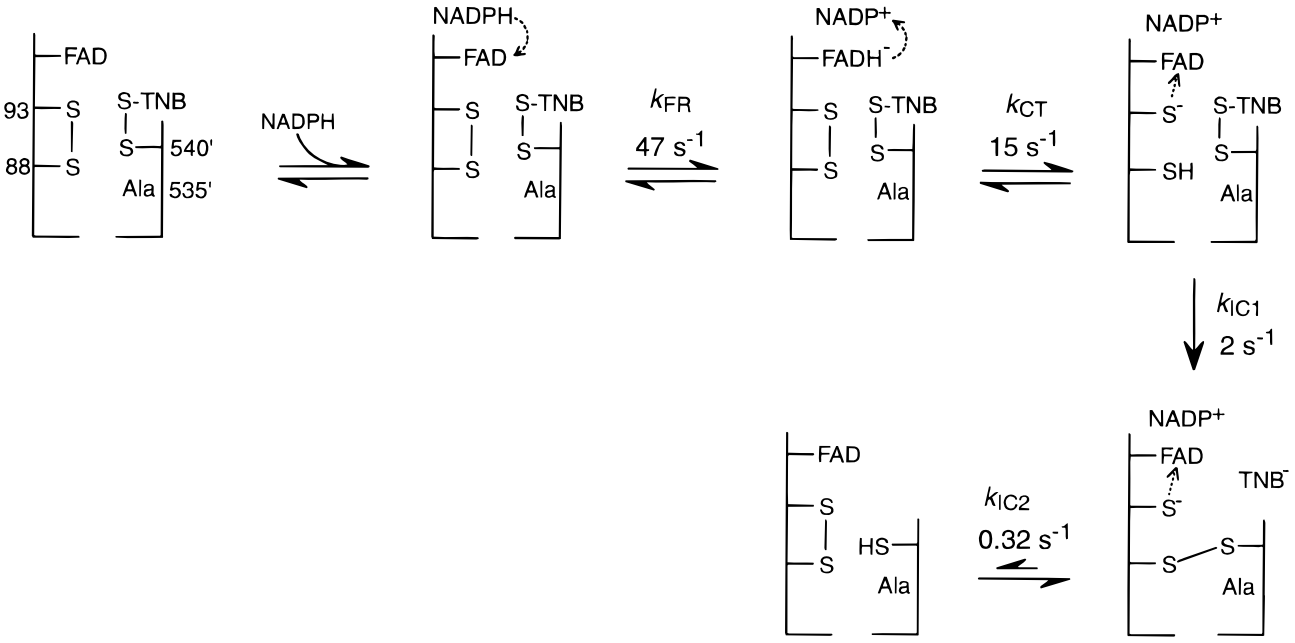
In the reductive titrations of C535−TNB and C540−TNB with NADPH, approximately 1 equiv of TNB anion was released before the formation of the thiolate−FAD charge transfer complex. In contrast, less than 0.1 equiv of TNB anion was released in the reductive titrations in the control experiments with DTNB-treated C535A/C540A and Mut-9 (cf. Figures 3 and 4). Reducing equivalents pass from NADPH, via the flavin and the redox active disulfide−dithiol, to the C-terminal mixed disulfide of C535−TNB or C540−TNB. The fact that the first reducing equivalent is taken up primarily by the mixed disulfide at equilibrium shows that the redox potential of the mixed disulfide is higher than the potentials of the FAD or the redox active disulfide−dithiol. The presence of two isosbestic points and absence of long-wavelength absorbance indicate that only two species are involved in the first phase: the oxidized enzyme before and after TNB release (Figure 3A and inset).

The fact that reducing equivalents are transferred from the active site dithiol to the C-terminal cysteines raises the question of whether the transfer is fast enough to be part of catalysis. Therefore, the rapid reaction kinetics of the reduction of C540−TNB by NADPH have been studied. We also investigated the reductive half-reaction of C535A which served as a control. Data analysis for the reaction of C535A with NADPH shows a clear path of reducing equivalents from NADPH to the FAD at a rate of approximately 190 $s^{-1}$ in forming the reduced flavin−$NADP^+$ charge transfer complex, followed by reduction of the active site disulfide by the reduced flavin at a rate of 47 $s^{-1}$ in forming the thiolate−FAD charge transfer complex (Figure 6).

The reduction of C540−TNB by 1 equiv of NADPH is more complex. Similar spectral changes were observed in the first two phases in comparison with the control experiment with C535A (Figure 7). Interchange between the active center dithiol and the TNB mixed disulfide provides two additional phases: formation of a new mixed disulfide with displacement of TNB anion followed by reformation of the active site disulfide leaving Cys540 as a free thiol (cf. Figures 6 and 7).

A working hypothesis mechanism for the reduction of C540−TNB is proposed in Scheme 2. It is well-known that the thiols of the active site cysteines have distinct functions in lipoamide dehydrogenase and glutathione reductase. One of them is the electron transfer thiol which interacts with the flavin and is nearer the carboxyl terminus, while another one is the thiol−disulfide interchange thiol (*1*, *11*, *30*, *31*). Therefore, we assume that Cys93 of *Pf*TrxR is the electron transfer thiol and that Cys88 is the thiol−disulfide interchange thiol on the basis of the corresponding assignment in lipoamide dehydrogenase and glutathione reductase. The enzyme form on the left represents C540−TNB. The first three steps of Scheme 2 apply also to the reduction of C535A

Scheme 2: Proposed Mechanism for the Reduction of C540−TNB by NADPH[a]




[a] The active site disulfide is made up of Cys88 (interchange thiol) and Cys93 (flavin-interacting thiol). The C-terminal disulfide is made up of Cys535 (here mutated to Ala) and Cys540. The dotted arrows indicate the direction of charge transfer. The acid−base catalyst present in other members of this enzyme family has been omitted for simplicity (*1*). The break in the polypeptide chain between the two disulfides indicates that the C-terminus of one subunit interacts with the active site of the other, by analogy to the closely related human thioredoxin reductase, glutathione reductase, and mercuric reductase (*2*, *11*, *18*, *34*). Preliminary evidence indicates that Cys540 is the C-terminal interchange thiol (P.-F. Wang, L. D. Arscott, and C. H. Williams, Jr., unpublished results).

(control). The observed rate constant for reduction of FAD by 1 equiv of NADPH is 47 $s^{-1}$ in the case of C540−TNB. The reduction of the active site disulfide proceeds at a rate of 15 $s^{-1}$. Thiol−disulfide interchange between the active site thiol (Cys88) and the C-terminal mixed disulfide follows at a rate of 2.4 $s^{-1}$ with the release of the free TNB anion. The active sites of lipoamide dehydrogenase, glutathione reductase, and mercuric reductase are composed of elements from both monomers (polypeptide chains) (*11*, *34*). It is proposed, on the basis of previous studies with mercuric reductase (*32*, *33*), that the thiolate from one monomer (Cys88) attacks the C-terminal cysteine (Cys540′ in the case of C540−TNB) from the other monomer of the dimer in the interchange reaction. During the interchange reaction, the thiolate of Cys93 still forms a thiolate−FAD charge transfer complex as suggested by the fact that there is little signal change in the long-wavelength band while the absorbance at 412 nm increases in this phase due to formation of free TNB anion. Finally, the last phase appears to be the second half of the thiol−disulfide interchange reaction in which the thiolate of Cys93 attacks the new mixed disulfide between Cys88 and Cys540′ formed in the previous phase. The observed rate constant of this phase is very low (0.32 $s^{-1}$) in this reduction with only 1 equiv of NADPH, and the reaction proceeds to completion very slowly (see Figure 3A).

Thioredoxin from *P. falciparum* has not been isolated. The turnover number of the wild type *Pf*TrxR has been determined therefore using 100 $\mu$M *E. coli* thioredoxin and 200 $\mu$M NADPH, and is quite slow (5 $s^{-1}$) at 37 °C (*15*). The observed rate of the interchange reaction between the active site thiolate of Cys88 and the C-terminal mixed disulfide of C540−TNB ($k_{IC1}$) is 2.4 $s^{-1}$ at 4 °C and is independent of the NADPH concentration (Table 3). Given the caveat that *E. coli* thioredoxin is used in the assay and applying a $Q_{10}$ of 2.4 applicable for other members of this family of enzymes (*22*), the rate of interchange $k_{IC1}$ would be 43 $s^{-1}$ at 37 °C. This would be fast enough to be part of turnover of the wild type enzyme which is 5 $s^{-1}$.

In conclusion, the evidence provided by this study supports the hypothesis that the C-terminal cysteines are in communication with the active site nascent dithiol. Both Cys535 and Cys540 are able to accept the reducing equivalents passed from the active site dithiol. This indicates that the communication between the C-terminal cysteines and the active site is probably a part of catalysis.

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