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MINIREVIEW

Thioredoxin reductase

Two modes of catalysis have evolved

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Thioredoxin reductase (EC 1.6.4.5) is a widely distributed flavoprotein that catalyzes the NADPH-dependent reduction of thioredoxin. Thioredoxin plays several key roles in maintaining the redox environment of the cell. Like all members of the enzyme family that includes lipoamide dehydrogenase, glutathione reductase and mercuric reductase, thioredoxin reductase contains a redox active disulfide adjacent to the flavin ring. Evolution has produced two forms of thioredoxin reductase, a protein in prokaryotes, archaea and lower eukaryotes having a M_r of 35 000, and a protein in higher eukaryotes having a M_r of 55 000. Reducing equivalents are transferred from the apolar flavin binding site to the protein substrate by distinct mechanisms in the two forms of thioredoxin reductase. In the low M_r enzyme, interconversion between two conformations occurs twice in each catalytic cycle. After reduction of the disulfide by the flavin, the pyridine nucleotide domain must rotate with respect to the flavin domain in order to expose the nascent dithiol for reaction with thioredoxin; this motion repositions the pyridine ring adjacent to the flavin ring. In the high M_r enzyme, a third redox active group shuttles the reducing equivalent from the apolar active site to the protein surface. This group is a second redox active disulfide in thioredoxin reductase from *Plasmodium falciparum* and a selenenylsulfide in the mammalian enzyme. *P. falciparum* is the major causative agent of malaria and it is hoped that the chemical difference between the two high M_r forms may be exploited for drug design.

Keywords: flavoprotein; thioredoxin; thioredoxin reductase; selenium, disulfide; dithiol; selenenylsulfide; redox active; ribonucleotide reductase; transcription factor activation; drug design.

With most enzymes, the structure, and the mechanism that is associated with it, are essentially the same regardless of the enzyme source, whether that be prokaryote, archaea or eukaryote, i.e. evolution has decided on one way to effect catalysis. However, there are enzymes where the same reaction is catalyzed by more than one structure and mechanism (e.g. methionine synthase [1]), and thioredoxin reductase is another such enzyme [2,3]. Thioredoxin reductase is a flavoprotein that catalyzes the reduction of thioredoxin by NADPH [4,5]. The substrate thioredoxin is a small protein of M_r 12 000 which in its dithiol state plays a key role in maintaining the redox environment of the cell [6]. Important functions of reduced thioredoxin include the reduction of nucleotides to deoxynucleotides and the modulation of transcription factors such as NF-κB in eukaryotes [6–8].

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Abbreviations: $AADP^+$, 3-aminopyridine adenine dinucleotide phosphate; SeC, selenocysteine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TNB, thionitrobenzoate; TCEP, Tris(2-carboxyethyl)phosphine.

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CHARACTERISTICS OF AN ENZYME FAMILY

Thioredoxin reductase is a member of the family of dimeric flavoenzymes that catalyze the transfer of electrons between pyridine nucleotides and disulfide/dithiol compounds and promote catalysis via FAD and a redox active disulfide (Table 1) [9–13]. The family includes lipoamide dehydrogenase, glutathione reductase and mercuric reductase, which vary only slightly in structure and mechanism depending on the source. In these three cases, catalysis has evolved in only one way. On the other hand, two distinct types of thioredoxin reductase have evolved [2,3]. Both are dimeric, and catalysis is again brought about by FAD and a redox active disulfide. Thioredoxin reductase isolated from *Plasmodium falciparum* and other higher eukaryotes has a subunit M_r of 55 000, like that of glutathione reductase and lipoamide dehydrogenase, while thioredoxin reductase from lower species, including lower plants and fungi, lacks a separate interface domain and has a subunit M_r of 35 000. The low M_r type from *Escherichia coli* was reviewed in 1995 and further features of its structure will be covered in the first part of this review [5]. We will then turn to recent work on the high M_r form from human placenta and from *P. falciparum*, the major causative agent of malaria [2,14,15].

The common chemistry effected by the disulfide reductase family of enzymes involves the transfer of reducing equivalents from the pyridine nucleotide substrate to the disulfide substrate.

Table 1. Characterization of an enzyme family: pyridine nucleotide-disulfide oxidoreductases.

Thioredoxin reductase (2 distinct types)	Lipoamide dehydrogenase and glutathione reductase
High M_r type	Subunit M_r approximately 55 kDa (all sources)
Subunit M_r approximately 55 kDa (human and *Plasmodium falciparum*)	
Dimeric proteins with one FAD, one redox active disulfide and a third redox active group in each subunit. The third group is a selenenylsulfide in human TrxR and a disulfide in the *P. falciparum* enzyme	
Low M_r type	Dimeric proteins with one FAD and one redox active disulfide in each subunit
Subunit M_r approximately 35 kDa (*E. coli* and other prokaryotes, yeast, mycoplasmas, *Giardia duodenalis*, *Arabidopsis thaliana* and *Methanococcus jannaschii*)	
Dimeric proteins with one FAD and one redox active disulfide in each subunit	

A hydride ion passes from the NAD(P)H to the flavin and the reducing equivalent is transferred from the reduced flavin to the active site disulfide. In the absence of an acceptor substrate, a thiolate–flavin charge transfer complex is stabilized as shown schematically for glutathione reductase and lipoamide dehydrogenase in the top line of Fig. 1 [9]. This CT complex is characterized by an absorbance peak around 540 nm [16]. The flavin reduction and its reoxidation by the disulfide require the apolar medium provided by the enzyme. The subsequent

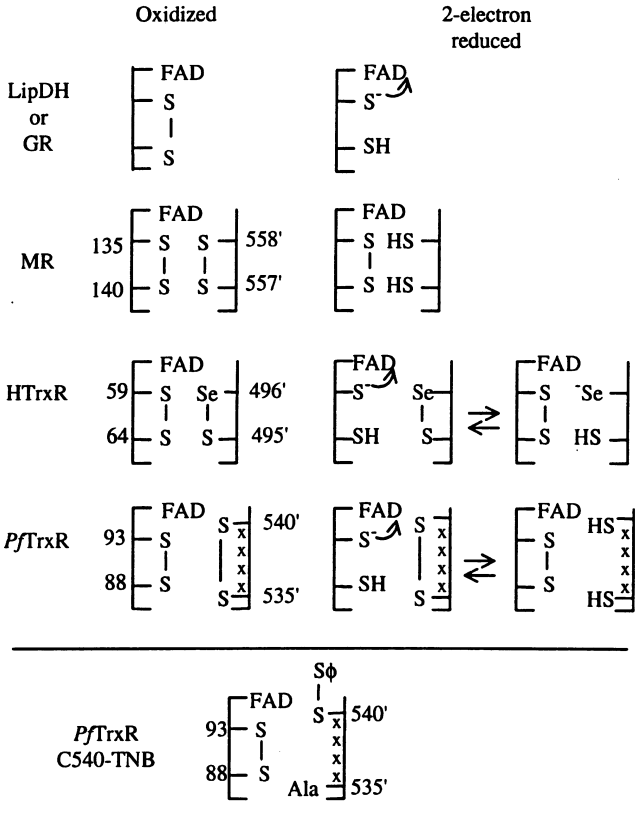

Fig. 1. Active sites in the oxidized and two-electron reduced states. The abbreviations are as follows: LipDH, lipoamide dehydrogenase; GR, glutathione reductase; MR, mercuric reductase from *Pseudomonas aeruginosa* Tn*501*; HTrxR, human thioredoxin reductase; and *Pf*TrxR, *Plasmodium falciparum* thioredoxin reductase. Residues having numbers without primes come from one subunit while those with primes come from the other; the break at the bottom also symbolizes the two polypeptide chains. The curved arrow indicates charge transfer from the donor thiolate to the acceptor FAD. The scheme below the line represents the mixed disulfide complex between Cys540 and TNB, where ϕS is the TNB anion.

dithiol–disulfide interchange between the nascent dithiol and the disulfide substrate takes place in a more polar medium.

The path of electrons in catalysis by glutathione reductase or lipoamide dehydrogenase is clear: from the nicotinamide ring of NAD(P)H on the *re* side of the isoalloxazine ring to the disulfide on the *si* side of FAD and on to the disulfide substrate [17,18]. (*Re* and *si* in nontechnical terms refer to specific sides of the isoalloxazine ring; the *re* side has the benzene ring on the left when the ribytyl side chain is at the bottom and the *si* side is the opposite side, e.g. in Fig. 2, S138 is on the *re* side.) Interchange between the nascent dithiol and the substrate disulfide is catalyzed by a nearby histidine residue [17,19]. Only one thiol can interact with the flavin (referred to as the flavin interacting thiol) and the remaining thiol can only participate in dithiol–disulfide interchange with the substrate (referred to as the interchange thiol) [17,20,21]. The milieu of the interchange thiol is more polar than that of the flavin interacting thiol [17].

In contrast, the path of electrons in low M_r *E. coli* thioredoxin reductase was not obvious from the initial crystal structure [22]. Both the disulfide and the pyridine nucleotide were on the *re* side of the flavin, with the disulfide adjoining the isoalloxazine ring of FAD but interposed between the nicotinamide ring of NADPH and the flavin. There was no histidine residue near the disulfide to catalyze dithiol–disulfide interchange. Instead, an Asp residue proved to be the catalyst [23]. Moreover, it did not appear possible for thioredoxin to undergo interchange with the reductase dithiol, which was buried near the flavin ring. A solution to these problems was suggested by a model that emulated the domain arrangement observed in glutathione reductase [24]. A large rotation of the NADPH domain relative to the FAD domain would move the redox active dithiol to the protein surface where it could react with its protein substrate, and would position the nicotinamide of NADPH parallel to the flavin ring. Thus, interconversion between the observed and model conformations was proposed to occur at two steps in the catalytic cycle. A considerable body of mechanistic evidence supports the idea that the two conformations are in equilibrium in solution [25–29]. The initial crystal structure is referred to as the FO conformation, because the flavin and the disulfide are juxtaposed for flavin oxidation. FR is the alternate rotated structure in which the flavin and the pyridine nucleotide are juxtaposed to allow flavin reduction [26].

STRUCTURE OF *E. COLI* THIOREDOXIN REDUCTASE IN THE FR CONFORMATION

The properties and reactivities of complexes of thioredoxin with thioredoxin reductase, stabilized by disulfide cross-links,

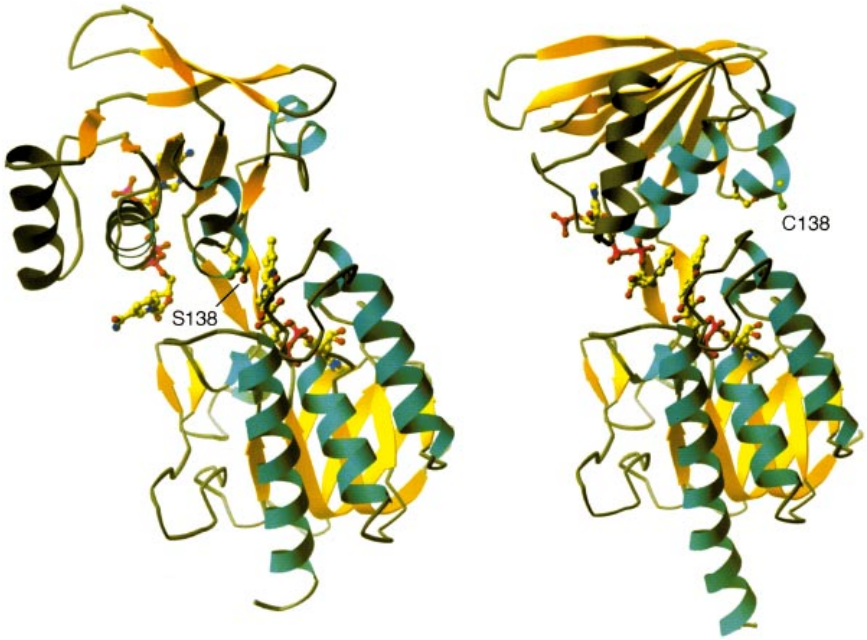

Fig. 2. Drawings of the FO (left) and FR (right) conformations of thioredoxin reductase, based on the crystal structures of C138S (FO) and C135S (FR) [24,30]. $NADP^+$ and $AADP^+$ are bound, respectively, in the two structures. The NADPH domain (top) rotates relative to the FAD domain in the conversion from FO to FR, as can be seen from the positions of the bound pyridine nucleotides and the gray helix, Nα2. The pyridine nucleotides, active site cysteines, and FAD prosthetic groups are shown in ball-and-stick representation. Only one subunit of the dimer is displayed, and the thioredoxin attached to Cys138 has been omitted from the FR structure to simplify the view. Thioredoxin interacts primarily with the NADPH domain but also contacts residues from the FAD domain. The bound pyridine nucleotide structures may represent either $NADP^+$ or NADPH.

have demonstrated that these complexes are locked into the FR conformation and do not undergo rotation to form the FO conformer [25]. The crystal structure of a stable disulfide complex has recently been determined (Fig. 2) [30]. In the observed FR structure the NADPH domain has rotated about 66° as postulated by model building [24]. Reorientation of the domains moves Cys138 away from the flavin to accommodate the binding and reaction with thioredoxin. The domain rearrangement simultaneously stacks the pyridine ring of a bound pyridine nucleotide analog, 3-aminopyridine adenine dinucleotide phosphate ($AADP^+$), against the isoalloxazine ring of the FAD in a position that would permit hydride transfer during catalysis if NADPH were bound instead.

Domain rotations thus solve the steric problems posed by the arrangement of active site sequences in the low M_r thioredoxin reductases from prokaryotes, lower eukaryotes, archaea, lower plants and fungi (Table 1). Both conformations of the enzyme are essential for completion of the catalytic cycle. The FR conformation is required for flavin reduction by NADPH and for reduction of the large protein substrate thioredoxin by the enzyme dithiol. The FO conformation is required for the transfer of electrons from the flavin to the enzyme disulfide [26].

THIOREDOXIN REDUCTASE FROM HIGHER EUKARYOTES

Turning now to the high M_r thioredoxin reductase from human placenta and from *P. falciparum*, we will see that a completely different mechanism has evolved to transfer reducing equivalents from the apolar interior to the disulfide substrate. Mammalian thioredoxin reductase was isolated first from rat liver in the seventies by Holmgren and associates, and found to have a M_r of 55 000, like that of glutathione reductase, and much higher than that of *E. coli* thioredoxin reductase [3]. Reduction of the rat liver enzyme with NADPH produced a spectrum having long wavelength absorbance typically seen when glutathione reductase or lipoamide dehydrogenase are reduced to form the thiolate–flavin charge transfer complex [16], and quite distinct from reduction of low M_r *E. coli* thioredoxin reductase in which the thiolate–FAD charge transfer complex is not stabilized and the two-electron reduced enzyme is an equilibrium mixture of FAD–dithiol and reduced flavin–disulfide forms [29]. Therefore, our hypothesis was that the mechanism of high M_r thioredoxin reductase would be like that of glutathione reductase, and quite distinct from low M_r thioredoxin reductase; this hypothesis has been shown to be correct but with a complication discussed below [2].

The identification of a residue of the unusual amino acid, selenocysteine (SeC) penultimate to the C-terminal Gly residue in human thioredoxin reductase by Stadtman and her colleagues was a seminal discovery [31–33]. Comparison of the C-terminal amino-acid sequences of two high M_r thioredoxin reductases with mercuric reductase, another member of this enzyme family, reveals interesting contrasts. The three enzymes have groups near their C-termini that are potentially redox active: Cys–SeC in human thioredoxin reductase, Cys–XXXX–Cys in *P. falciparum* thioredoxin reductase and Cys–Cys in mercuric reductase (Fig. 1). The C-terminal sequences are SGASILQAGCUG in thioredoxin reductase from human placenta [32,33], AAKGGCGGGKCG in thioredoxin reductase from *P. falciparum* [34], and FNKDVKQLSCCAG in mercuric reductase from *Pseudomonas aeruginosa* Tn *501* [35,36], where U = SeC.

The C-terminal amino acids from one monomer of mercuric reductase are adjacent to the active site disulfide of the second monomer and it has been shown that dithiol–disulfide interchange can occur between the nascent active site dithiol and the C-terminal disulfide [37,38]. Extensive homology between high M_r thioredoxin reductase and mercuric reductase in all domains suggests that a similar structure is present in the three enzymes as depicted in Fig. 1. It has been widely assumed that the C-terminal groups in thioredoxin reductase must be functional in reaction with thioredoxin and it has been our hypothesis that they constitute evolution's second way of transferring reducing equivalents from the apolar active site to the enzyme surface. Alkyl hydroperoxide reductases also utilize a second redox active disulfide, but in this case it is at the N-terminus rather than the C-terminus [39].

To demonstrate that the C-terminal groups are directly functional in catalysis requires at least three steps: first to show that they are redox active; second to show that they communicate with the redox active nascent dithiol at the active site; and third to show that the communication is at a rate commensurate with catalysis. It has been shown that site-directed mutagenesis of either C-terminal Cys residue in *P. falciparum* thioredoxin reductase, or of the SeC in human thioredoxin reductase leads to loss of activity, suggesting that they are functional [40–42]. Chemical or enzymatic modification affecting the C-terminal residues also lead to loss of activity but only when the redox active residues are in the reduced state [14,43–48]. Earlier work has demonstrated that the Cys-SeC of the mammalian thioredoxin reductase is redox active and communicates with the active site dithiol [2].

The redox states for the groups in the C-terminal sequence of *P. falciparum* thioredoxin reductase are a disulfide in the oxidized state and a dithiol in the two-electron reduced state. The Cys–SeC bridge in the human enzyme is a selenenylsulfide in its oxidized state and, in the reduced state, a thiol and a selenolate as suggested by its much lower pK_a [49] (Fig. 1).

Ultimately, comparison of thioredoxin reductase isolated from mammals with that from a causative agent of malaria, *P. falciparum*, will reveal differences that may contribute to rational drug design. For rational drug design to be effective, the enzyme from the two sources must be different in some significant way. In this regard, evolution seems to be on our side because there are very considerable chemical differences between sulfur and selenium, and the redox active residues are separated by four residues in one species and are adjacent in the other. Promising compounds will be required that react maximally with the parasite enzyme and minimally with the human thioredoxin reductase.

TITRATION WITH NADPH OR DITHIONITE SHOWS THE THIRD REDOX ACTIVE GROUP IN HIGH M_R THIOREDOXIN REDUCTASE

Figure 1 will be used to discuss the dithionite and NADPH titration data. FAD and the active site disulfide/dithiol that are common to all members of this enzyme family are shown on the left, and the C-terminal disulfide/dithiol, the putative new part of the active site, is on the right. The break in the polypeptide chain signifies that the groups on the left come from one subunit (residue numbers without primes) and the C-terminal disulfide or selenenylsulfide (residue numbers with primes) is contributed by the other subunit, as is confirmed in the case of mercuric reductase [37]. Titration with dithionite gives a wealth of information that assists in the interpretation of the NADPH data. NADPH reduces glutathione reductase only to the two-electron reduced state, the thiolate–flavin charge transfer complex. Dithionite, being a strong reductant, first reduces the enzyme to the thiolate–flavin charge transfer complex but then further reduces the enzyme FAD with a second two-electron equivalent. The conclusion is that glutathione reductase has only two redox active groups, FAD and the active site disulfide/dithiol [9]. Our understanding of the dithionite titration of high M_r thioredoxin reductase (see below) depended totally on having a precedent. Mercuric reductase shows a pattern of reduction which is 'diagnostic' for the presence of an additional redox active group: it is completely reduced in three phases. The product after the first two-electron equivalent is shown in Fig. 1 with all of the first equivalent taken up by the C-terminal disulfide, demonstrating that its redox potential is higher than that of the FAD or the active site disulfide [38]. The resulting dithiol binds mercuric ion as it moves into the active site for reduction; as the dithiol does not reoxidize, its function is different from that proposed for the groups in thioredoxin reductase which are redox active in catalysis [50].

It should be said at the outset that there are features of the dithionite titrations of high M_r thioredoxin reductase that we do not understand; what follows is a preliminary interpretation. The dithionite titration of the *P. falciparum* enzyme is shown in Fig. 3. Approximately 2 eq. of dithionite were required for full thiolate–flavin charge transfer formation, in contrast to only one equivalent required in the titration of glutathione reductase. Thus, a third redox active group is present; the C-terminal disulfide is redox active. The first six spectra detected during this phase (540 nm absorbance increase) displayed a set of four isosbestics suggesting that only two enzyme species were

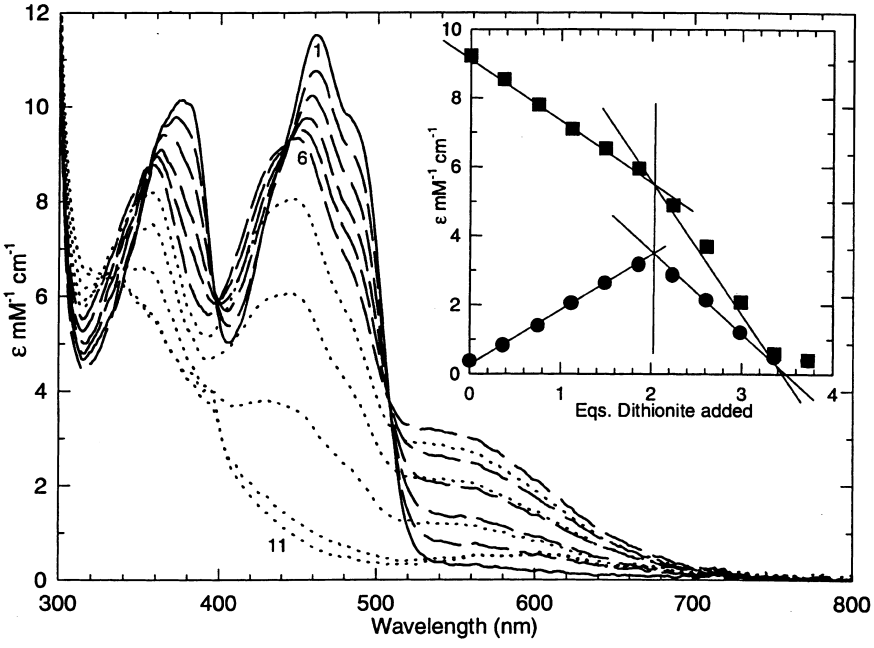

Fig. 3. Reduction of thioredoxin reductase from *P. falciparum* by dithionite. The oxidized enzyme is shown in spectrum 1, fully formed thiolate–flavin charge transfer complex is spectrum 6 and fully reduced enzyme is spectrum 11. The peaks at 380, 395 and 620 nm in the final two spectra are due to methyl viologen radical, the electron mediator. The inset shows the data at 540 nm (circles) and at 490 nm (squares). Spectra 1–6 were analyzed by singular value decomposition using SPECFIT Analysis software (Spectrum Associates, Chapel Hill, NC, USA).

detectable. Nevertheless, the spectra were analyzed by singular value decomposition. Two of the three eigenvectors observed clearly represented oxidized enzyme and fully formed thiolate–flavin charge transfer complex (maximally formed when 2 eq. of dithionite had been added); it was assumed that the third eigenvector could be ascribed to the equilibrium mixture shown in Fig. 1. Mixed disulfides that are undoubtedly intermediates in the dithiol–disulfide interchange reaction need also to be considered (see below) [2]. The second part of the titration of the parasite enzyme, reduction of the thiolate–FAD charge transfer complex (540 nm absorbance decrease), required one equivalent of dithionite, as expected based on the behavior of the human enzyme (see below) [2] and of glutathione reductase [9]. The study presented in Fig. 3 has not been published and will be presented in a more complete form elsewhere.

There are differences between the parasite and human [2] enzymes that will require further investigation. However, both took at least 2 eq. of dithionite to fully form the thiolate–flavin charge transfer complex, indicating that reducing equivalents are shared between the active site disulfide and the C-terminal selenenylsulfide in the case of the human enzyme and the C-terminal disulfide in the parasite enzyme (Fig. 1). The second part of the titration of human thioredoxin reductase, reduction of the thiolate–FAD charge transfer complex, requires one equivalent of dithionite just as it does with glutathione reductase [2]. The qualitative differences observed in the dithionite titrations of human and *P. falciparum* thioredoxin reductase may reflect the chemistry of selenium vs. sulfur and give hope for rational drug design.

SOME PROPERTIES OF THE C-TERMINAL DISULFIDE-DITHIOL: THE THIRD CATALYTIC GROUP IN THIOREDOXIN REDUCTASE FROM *P. FALCIPARUM*

Thioredoxin reductase from *P. falciparum* has been cloned and can be expressed in *E. coli* [34,51]. (Until very recently it had not been possible to express the mammalian enzyme in *E. coli* because of the SeC, and even now fully active recombinant enzyme is not yet available [42,52–54].) As mentioned above, mutagenesis of either Cys residue comprising the C-terminal disulfide present in *P. falciparum* thioredoxin reductase led to loss of activity [41]. The two modified forms of the parasite enzyme, C535A having Cys540 remaining and C540A having Cys535 remaining, have been used to confirm that the C-terminal group is redox active and that it interacts with the active site dithiol [15]. The thiol in each mutant enzyme was reacted with DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] yielding a mixed disulfide (Fig. 1, bottom). The thionitrobenzoate (TNB) anion when released, absorbed strongly and showed that the reaction was stoichiometric. C535A resulted in the more reactive construct referred to as C540-TNB (Fig. 1). With these constructs, we can pose the question of whether the active site dithiol, having been reduced by NADPH via the flavin, can interchange with the C-terminal mixed disulfide, all signaled by the release of a TNB anion. NADPH reduced the FAD; the $FADH^-$ in turn reduced the active site disulfide; the resulting dithiol interchanged with the C-terminal mixed disulfide to release a TNB anion and form a new mixed disulfide between the interchange thiol, Cys88 and Cys540. The dithiol–disulfide interchange was completed by the attack of the flavin interacting thiolate, Cys93 on the new mixed disulfide to reform the active site disulfide and Cys540 [15]. Titration of Cys540-TNB in the mutant enzyme C535A with NADPH showed the release of one equivalent of TNB anion as the first equivalent of NADPH reacted. As the TNB anion is an excellent leaving group, the interchange was complete, in contrast to the titration of wild-type enzyme where dithiol–disulfide interchange between the active center dithiol and the C-terminal disulfide reached equilibrium. After release of the TNB anion, the thiolate–flavin charge transfer complex formed as the second equivalent of NADPH reacted. Thus, reducing equivalents entering the mutant enzyme through the flavin can reach the mixed disulfide in the C-terminal sequence via the active site disulfide.

Tris(2-carboxyethyl)phosphine (TCEP), is a relatively specific reagent for disulfide reduction [55]. TCEP reacts with a model intramolecular disulfide to form a thiophosphonium moiety and a thiolate; the reaction is completed by hydrolysis of the thiophosphonium moiety. Because this reaction is reversible, the back reaction competes with hydrolysis of the thiophosphonium moiety. The model reaction is therefore carried out at lower pH so that protonation of the thiolate inhibits the back reaction.

TCEP was used to reduce the C-terminal disulfide of *P. falciparum* thioredoxin reductase and to determine if the resulting thiol would interchange with the active site disulfide. TCEP reacted slowly with the exposed C-terminal disulfide of wild-type thioredoxin reductase from *P. falciparum*; reducing equivalents were transferred to the active site disulfide to form the thiolate–flavin charge transfer complex which was detected spectrally. Thus in the enzyme, the back reaction (elimination of the thiophosphonium moiety) was not observed due to the competing reaction of the thiolate with the nearby active site disulfide. Glutathione reductase from yeast with its buried active site disulfide did not react with TCEP nor did a mutant of *P. falciparum* thioredoxin reductase lacking the C-terminal thiols. The spectra detected during formation of the thiolate–flavin charge transfer complex were analyzed by singular value decomposition. Two of the three eigenvectors could obviously be ascribed to oxidized enzyme and the fully formed thiolate–flavin charge transfer complex, while the third eigenvector was assumed to be the mixed disulfide intermediate [2]. The charge transfer complex formed in the reaction with TCEP was identical to that produced by dithionite reduction (Fig. 3, spectrum 6).

TCEP reacted with the selenenylsulfide of the human enzyme rapidly in contrast to the disulfide of thioredoxin reductase from *P. falciparum* which reacted slowly with the reagent. The thiolate–flavin charge transfer complex was the product with the human enzyme, as with the enzyme from the parasite. These data show that the C-terminal disulfide or selenenylsulfide, when reduced by TCEP, can interchange with the active site disulfide as shown by formation of the thiolate–flavin charge transfer complex. Kinetics of the reduction of the two thioredoxin reductases by TCEP show that the reactivities of the selenenylsulfide and the disulfide are different. It is clear from these data, and the possible difference in redox potential between the two C-terminal redox groups (suggested by their reactivity with the nascent active site dithiol), that the two groups give the human and parasite enzymes distinct properties.

REDUCTION OF THIOREDOXIN REDUCTASE FROM *P. FALCIPARUM* WITH NADPH: RAPID REACTION KINETICS

The reduction of thioredoxin reductase from *P. falciparum* by NADPH suggested that the rate of dithiol–disulfide interchange with the group in the C-terminal sequence may be catalytically competent. Figure 4 shows the kinetics of thiolate–flavin charge

transfer complex formation when the enzyme was reduced by excess NADPH. The reductive half reaction of an altered enzyme form lacking the C-terminal disulfide is shown for comparison and it can be seen that it has a much simpler pattern. The same charge transfer absorbance was formed with both enzymes. Whereas in the wild-type enzyme five exponential phases were required to fit the data and the reaction was not complete in 400 ms, in the mutant enzyme only two exponential phases were required to fit the data and the reaction was completed in 100 ms. Given that dithiol–disulfide interchange takes place in two steps, formation and breakdown of mixed disulfide, and given that 2 eq. of NADPH are required, five exponential phases were not surprising. It is our hypothesis that at least some of the extra phases seen in the reaction of the wild-type enzyme but not in the mutant can be attributed to interchange between the active site dithiol and the C-terminal disulfide. The experiment presented in Fig. 4 has not been published and will be presented in a more complete form elsewhere.

Are the rates of dithiol–disulfide interchange in the reductive half reaction rapid enough to be catalytically competent? The substrate for *P. falciparum* thioredoxin reductase has only very recently been identified (S. Müller and K. Becker, independently, unpublished results). The turnover number has been determined with *P. falciparum* thioredoxin in the presence of 30 μM insulin (which was included because of the low K_m for Trx) at temperatures from 4 °C (the temperature of the rapid reaction kinetics) to 37 °C. For example, the turnover number at 4 and 25 °C is 340 min^{-1} and 1960 min^{-1}, respectively; the apparent K_m for Trx is approximately 3 μM and the activation energy is 12.7 kcal/mole (53 $kJ \cdot mol^{-1}$). The steady-state kinetics have also been determined in direct assays in the absence of insulin, yielding a $K_m = 10.6$ μM (S. Kanzok and K. Becker, personal communication). The rates ascribed to dithiol–disulfide interchange in the reductive half reaction are approximately 3.5 times faster than the rate of turnover and are thus competent.

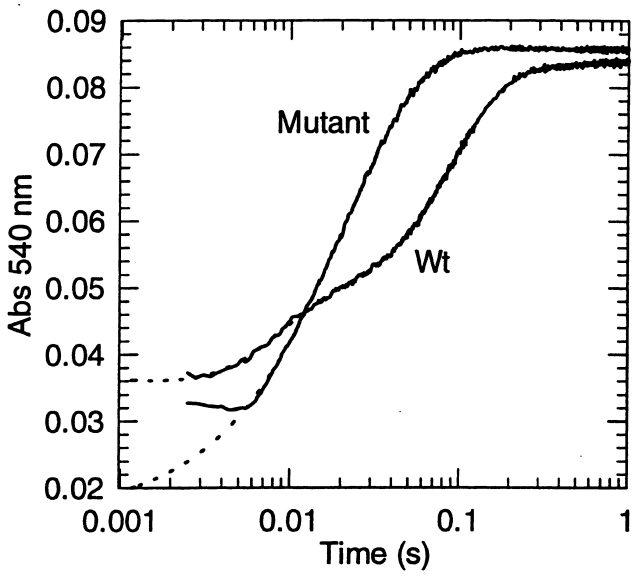

Fig. 4. Reduction of thioredoxin reductase from *P. falciparum* by NADPH: rapid reaction kinetics. Wild-type enzyme (14.0 μM) mixed with 30 eq. of NADPH; the data were fitted to the sum of five exponentials. Enzyme mutated to disable the disulfide in the C-terminal sequence (15.3 μM) mixed with 30 eq. of NADPH; the data were fitted to the sum of two exponentials.

CONCLUSIONS

The data presented here lead to the conclusion that two ways to catalyze the reduction of thioredoxin have evolved; more exactly, two means of transferring reducing equivalents from the apolar active site to the protein surface have developed. In lower species, rotation of one domain relative to the other brings the nascent dithiol from its position adjacent to the flavin to the protein surface for reaction with thioredoxin, while in higher organisms a third redox active group acts as a shuttle between the interior and exterior of the enzyme. This is in contrast to glutathione reductase and lipoamide dehydrogenase where the substrates are small enough to be bound adjacent to the interchange thiol. The differences between human and *P. falciparum* thioredoxin reductase give hope that rational drug design will be possible.

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