The Mechanism of High $M_r$ Thioredoxin Reductase from *Drosophila melanogaster*^*a*

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Drosophila melanogaster thioredoxin reductase-1 (DmTrxR-1) is a key flavoenzyme in ditteran insects, where it substitutes for glutathione reductase. DmTrxR-1 belongs to the family of dimeric, high $M_r$ thioredoxin reductases, which catalyze reduction of thioredoxin by NADPH. Thioredoxin reductase has an N-terminal redox-active disulfide (Cys$^{57}$–Cys$^{62}$) adjacent to the flavin and a redox-active C-terminal cysteine pair (Cys$^{489}$–Cys$^{490}$ in the other subunit) that transfer electrons from Cys$^{57}$–Cys$^{62}$ to the substrate thioredoxin. Cys$^{489}$–Cys$^{490}$ functions similarly to Cys$^{535}$–Sec$^{496}$ (Sec = selenocysteine) and Cys$^{535}$–XXX–Cys$^{490}$ in human and parasite *Plasmodium falciparum* enzymes, but a catalytic redox center formed by adjacent Cys residues, as observed in DmTrxR-1, is unprecedented. Our data show, for the first time in a high $M_r$ TrxR, that DmTrxR-1 oscillates between the 2-electron reduced state, $E_{H_2}$, and the 4-electron state, $E_{H_4}$, in catalysis, after the initial priming reduction of the oxidized enzyme ($E_{ox}$) to $E_{H_4}$. The reductive half-reaction consumes 2 eq of NADPH in two observable steps to produce $E_{H_4}$. The first equivalent yields a FADH$^+$–NADP$^+$ charge-transfer complex that reduces the adjacent disulfide to form a thiolate-flavin charge-transfer complex. $E_{H_4}$ reacts with thioredoxin rapidly to produce $E_{H_2}$. In contrast, $E_{ox}$ formation is slow and incomplete; thus, $E_{H_2}$ of wild-type cannot reduce thioredoxin at catalytically competent rates. Mutants lacking the C-terminal redox center, C489S, C490S, and C489S/C490S, are incapable of reducing thioredoxin and can only be reduced to the high molecular weight type, having a $M_r$ of 54,000–58,000, which contrasts with the well studied TrxR of *Escherichia coli* with a $M_r$ of 34,000. There is an interesting difference in the mechanism whereby these enzymes transfer reducing equivalents from the protein interior to the enzyme surface where Trx is bound and reduced. In low $M_r$ TrxR, one domain rotates from a conformation in which the redox-active disulfide is reduced by the flavin in the apolar interior to a conformation in which the nascent dithiol is carried to the hydrophilic surface of the enzyme, where it can reduce bound Trx. In this unusual mechanism, when the dithiol is near the surface to reduce Trx, the NADPH is in position to reduce the FAD (2). High $M_r$ thioredoxin reductases, on the other hand, have a second redox-active disulfide or selenosulfide pair that shuttles reducing equivalents from the nascent dithiol that is near the flavin to Trx bound at the surface (3).

High $M_r$ TrxRs are part of the disulfide reductase family that includes lipoylamine dehydrogenase, glutathione reductase, mercucur reductase, trypanothione reductase, and peroxiredoxin reductase. All of these flavoenzymes are homodimers (4), and each monomer comprises three domains: a flavin binding domain, a pyridine nucleotide binding domain, and a domain that provides an interface between the two monomers, as shown in Scheme 1. Each active site of thioredoxin reductase contains FAD and a so-called N-terminal redox-active disulfide (Cys$^{57}$ and Cys$^{62}$ in the *D. melanogaster* enzyme), both in the flavin-binding domain of one polypeptide chain, and a C-terminal redox disulfide (Cys$^{489}$ and Cys$^{490}$ in the *D. melanogaster* enzyme) originating from the other polypeptide chain (5, 6). This module operandi of using both monomers to carry out catalysis, which is common to all members of this enzyme family, is symbolized in Scheme 1 by the use of primes. The approach also requires His$^{644}$ from the adjacent subunit as the active site base involved in catalysis of the dithiol-disulfide interchange between the N-terminal Cys$^{57}$–Cys$^{62}$ pair and the C-terminal Cys$^{489}$–Cys$^{490}$ pair. Thus, the path of reducing equivalents in catalysis is from NADPH to FAD, from the reduced flavin to the charge transfer; Dm, *D. melanogaster*; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); $E_{ox}$, $E_{H_2}$, $E_{H_4}$, oxidized, 2-, 4- and 6-electron-reduced states of DmTrxR-1; eq, amount of a compound equivalent to one enzyme subunit; G6P, glucose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; Sec, selenocysteine; TNB, 5-thio-2-nitrobenzoate; Trx, thioredoxin; MV, methyl viologen.

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*The abbreviations used are: TrxR, thioredoxin reductase; CT, (Trx), a 12-kDa protein. The thioredoxin system is widely distributed in nature, and in most organisms it functions in concert with the glutathione system. However, because insects such as *Drosophila melanogaster* have no glutathione reductase, thioredoxin is reduced non-enzymatically by reduced Trx (1); thus, TrxR serves an additional role in insects. TrxR from higher eukaryotes, including *D. melanogaster*, is of the high molecular weight type, having a $M_r$ of 54,000–58,000, which contrasts with the well studied TrxR of *Escherichia coli* with a $M_r$ of 34,000. There is an interesting difference in the mechanism whereby these enzymes transfer reducing equivalents from the protein interior to the enzyme surface where Trx is bound and reduced. In low $M_r$ TrxR, one domain rotates from a conformation in which the redox-active disulfide is reduced by the flavin in the apolar interior to a conformation in which the nascent dithiol is carried to the hydrophilic surface of the enzyme, where it can reduce bound Trx. In this unusual mechanism, when the dithiol is near the surface to reduce Trx, the NADPH is in position to reduce the FAD (2). High $M_r$ thioredoxin reductases, on the other hand, have a second redox-active disulfide or selenosulfide pair that shuttles reducing equivalents from the nascent dithiol that is near the flavin to Trx bound at the surface (3).

High $M_r$ TrxRs are part of the disulfide reductase family that includes lipoylamine dehydrogenase, glutathione reductase, mercucur reductase, trypanothione reductase, and peroxiredoxin reductase. All of these flavoenzymes are homodimers (4), and each monomer comprises three domains: a flavin binding domain, a pyridine nucleotide binding domain, and a domain that provides an interface between the two monomers, as shown in Scheme 1. Each active site of thioredoxin reductase contains FAD and a so-called N-terminal redox-active disulfide (Cys$^{57}$ and Cys$^{62}$ in the *D. melanogaster* enzyme), both in the flavin-binding domain of one polypeptide chain, and a C-terminal redox disulfide (Cys$^{489}$ and Cys$^{490}$ in the *D. melanogaster* enzyme) originating from the other polypeptide chain (5, 6). This module operandi of using both monomers to carry out catalysis, which is common to all members of this enzyme family, is symbolized in Scheme 1 by the use of primes. The approach also requires His$^{644}$ from the adjacent subunit as the active site base involved in catalysis of the dithiol-disulfide interchange between the N-terminal Cys$^{57}$–Cys$^{62}$ pair and the C-terminal Cys$^{489}$–Cys$^{490}$ pair. Thus, the path of reducing equivalents in catalysis is from NADPH to FAD, from the reduced flavin to the
N-terminal disulfide, from the newly formed dithiol pair to the C-terminal redox pair on the other subunit, and finally, to bound Trx (Scheme 1) (4).

The C-terminal redox group used depends on the source of the TrxR. In TrxR from mammals a Cys-Sec (Sec = selenocysteine) sequence motif is present (7, 8), whereas in the enzyme from the malaria parasite Plasmodium falciparum, a cysteine pair is separated by a spacer of four amino acids (9, 10). However, DmTrxR-1 has adjacent cysteines (Cys\(^{489}\) and Cys\(^{490}\)) (1).

The thioredoxin reductases from human and P. falciparum have been the subject of intense investigations (5, 6, 10) and have been the subject of intense investigations (5, 6, 10). To fully understand the mechanism of this family of enzymes, it is important to know the redox states involved in catalysis. For this purpose we have analyzed the redox behavior of functional wild-type TrxR, as well as the three C-terminal mutants, C489S, C490S, and C489S/C490S. We provide evidence that these directly adjacent cysteines form a dithiol-disulfide redox center in DmTrxR that functions similarly to the Sec-Cys pair that is essential for catalysis in mammalian TrxR (14).

It has long been known that, in catalysis, lipopentadienogen and glutathione reductase utilize the \(E_{m}\) state and a 2-electron reduced state, \(EH_2\) (4, 15, 16), whereas low \(M\) TrxR functions via \(EH_2\), and a 4-electron reduced state, \(EH_4\) (2). In this paper, we present evidence that DmTrxR oscillates between the \(EH_2\) and \(EH_4\) states in catalysis, after the initial priming reduction of the oxidized enzyme (\(E_{m}\)) to the \(EH_2\) state. We also provide evidence that Cys\(^{489}\) in the C-terminal pair is involved in dithiol-disulfide interchange with Cys\(^{497}\) in the N-terminal pair.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant DmTrxR-1 and DmTrxR-1 mutant forms, C489S, C490S, and C489S/C490S, as well as DmTrx-2, were prepared as His-tagged proteins from *E. coli* and purified over a nickel-nitriol-triacetic acid column as previously described (1, 17). All reagents and other enzymes were obtained from Serva and Sigma and were of highest available purity. Titrations and stopped-flow experiments were conducted under anaerobic conditions using methods described previously (18), and all reactions were conducted in 100 mM potassium phosphate buffer, pH 7.0.

**Determination of Free Thiol Groups**—Free thiol groups were determined by reaction with DTNB, which was added in at least 5-fold excess. The number of free thiols of TrxR was calculated by the absorbance of the released TNB anion (\(A_{412\text{nm}} = 13.6 \text{ mM}^{-1}\text{ cm}^{-1}\)). Determinations of total thiol groups in enzymes were carried out under denaturing conditions by incubating with 8 M guanidinium hydrochloride before adding Ellman’s reagent.

**Reduction of DmTrxR Enzymes with NaBH\(_4\)**—Wild-type DmTrxR and DmTrxR mutants can be reduced rapidly by \(-100\)-fold excess sodium borohydride (100 mM in 0.02 M NaOH) under anaerobic conditions. Because borohydride is completely hydrolyzed at neutral or acidic pH within a few minutes, enzyme reduced in this manner could be directly used for titrations and stopped-flow experiments without concern about excess reductant being present.

**NAPDH-regenerating System**—In experiments in which it was desirable to have no significant quantities of NAPDP\(^*\) present, an NAPDH-regenerating system consisting of G6P and G6PDH was applied. To a mixture of 200 milliliters of G6PDH and 18 \(\mu\)M DmTrxR-1, 1 mM G6P and 6 \(\mu\)M NAPDP were added from the sidearm of the anaerobic cuvette. The reduction was followed spectrophotometrically over 2 h.

**Xanthine/Xanthine Oxidase System**—DmTrxR-1 was mixed in an anaerobic cuvette with \(-200\) \(\mu\)M xanthine, and 50 \(\mu\)M methyl viologen was added as an electron shuttle. To start the reaction, 10 \(\mu\)M of xanthine oxidase \((A_{260\text{nm}} = 0.4)\) was added from the side arm of the cuvette and the reaction was monitored spectrophotometrically for 1–3 h (19).

**EDTA/Light**—DmTrxR-1 in 0.1 M potassium phosphate buffer, 9 mM EDTA, pH 7.0, was mixed with 0.1–1 eq of methyl viologen. After making the solution anaerobic, the cuvette was irradiated with light (unfocused SunGun tungsten-halogen lamp, rheostat \(-80\) V, 15 cm from the sample) while keeping the temperature constant with a water bath. The photoreduction was monitored spectrophotometrically (20).

**Ananer Titrations**—Titrations were conducted in an anaerobic cuvette with an attached Hamilton syringe that allowed the exact addition of the reaction partner (16).

**Rapid Reaction Kinetics**—The rapid reaction kinetics studies were all conducted in a Hi-Tech SF-61 DX2 stopped-flow photometer under anaerobic conditions at 10 °C. The DmTrxR concentration was 10–15 \(\mu\)M after mixing. The data of the kinetic traces were analyzed by curve fitting with multiple exponential functions in a program written by Rong Chang, Chung-yen Chiu, and D. P. Ballou (University of Michigan, Ann Arbor, MD). The analysis methods are based on the Marquardt algorithm for solving differential equations (21).

The reaction of NADPH with \(E_{m}\) was carried out in the stopped-flow photometer. Time-dependent spectra were followed using the diode array detector, and single wavelength kinetic traces were recorded with a photomultiplier. The kinetic traces at 460, 440, 505, and 540 nm were recorded and, in the case of the reductive half-reaction, also at 360 nm to follow NADPH consumption at an isosbestic point between \(E_{m}\) and \(EH_4\). For the oxidative half-reaction, enzyme was reduced with NaBH\(_4\) and then reacted with oxidized DmTrx-2 in the stopped-flow instrument.

**RESULTS**

**Reduction of Wild-type DmTrxR-1 by NADPH**—Titration of DmTrxR-1 with its reducing substrate NADPH (\(K_w \approx 4.5 \mu\)M suggesting that binding is tight; see below) led to spectral changes that are similar to those of other flavin-dependent disulfide oxidoreductases (Fig. 1A) (4, 12, 16, 18). Reduction of DmTrxR-1 was detected as a decrease in the flavin absorbance maximum (\(\epsilon_{462\text{nm}} = 11.9 \text{ mM}^{-1}\text{ cm}^{-1}\) for \(E_{m}\)), and this was accompanied by an increase in absorbance around 540 nm that was caused by a charge-transfer interaction between the thiolate of Cys\(^{497}\) and the isoalloxazine ring. Two sharp isosbestic points (433 and 507 nm) were identified in the first phase that consumed 1 eq of NADPH (spectra 1–5). This indicated the predominance of two enzyme species, \(E_{m}\) and the equilibrium mixture of \(EH_2\) and \(EH_4\) shown in Scheme 2. The maximal charge-transfer absorbance (\(\epsilon_{540\text{nm}} = 2.6 \text{ mM}^{-1}\text{ cm}^{-1}\)) was not observed until 2 eq of NAPDP were added (inset), leading to the formation of \(EH_4\). It should be pointed out that the maximum CT absorbance value in the titration, in which equilibrium is reached after each addition of NADPH, is significantly lower than the absorbance observed after a single addition of excess NAPDP (\(\epsilon_{540\text{nm}} = 4.0 \text{ mM}^{-1}\text{ cm}^{-1}\)) (shown below).

Spectrum 8 in Fig. 1A resulted from the consumption of 2 eq of NAPDP. Because the FAD is still substantially oxidized, the high absorbance at 460 nm indicates that there are two redox centers in addition to the flavin. The first is the N-terminal redox-active disulfide with Cys\(^{497}\) contributing the flavin-interacting thiolate and Cys\(^{497}\) containing the interchange thiol. The other redox center is a disulfide made up of residues Cys\(^{489}\) and Cys\(^{490}\) in the C-terminal extension of the other subunit (Scheme 1).

The addition of more than 2 eq of NAPDP in the titration leads to small further decreases in the flavin absorbance at 460 nm and to a loss of the charge-transfer absorption at 540 nm. Both of these spectral effects are the result of the reduction of the FAD as a small amount of \(EH_4\) is formed (presumably from \(EH_2\), Scheme 2). Addition of excess NAPDP, however, does not result in complete reduction, because of the presence of NAPDP\(^*\). We therefore used more forcing conditions by coupling the reaction to an NAPDP-regenerating system consisting of G6P and G6PDH to eliminate NAPDP\(^*\) from the equilibrium (Fig. 1B). Indeed, this resulted in fully reducing the enzyme to \(EH_4\) as shown by the decrease in absorbance at 460 nm and complete loss of the 540 nm absorbance. \(EH_4\) was very unstable, and enzymatic activity was completely lost in a few minutes. The mechanism of this inactivation is not known. However, the results described below show that the redox potential for the formation of \(EH_4\) is considerably lower than that of free FAD, implying that the FADH\(^*\) is bound much less tightly.
than is FAD to DmTrxR-1. Thus, loss of activity could be associated with loss of FAD in the $E_{H6}$ form of DmTrxR-1.

Reoxidation by Ferricyanide—DmTrxR-1 can be conveniently reduced by sodium borohydride, as noted under “Experimental Procedures.” Although NaBH₄ is a strong reductant, it does not reduce DmTrxR-1 to $E_{H6}$, but rather to a reduced enzyme species with strong CT absorption ($\epsilon_{540 \text{ nm}} = 3.3 \text{ mm}^{-1} \text{ cm}^{-1}$) that is similar to the $E_{H4}$ spectrum obtained from reduction with NADPH ($\epsilon_{540 \text{ nm}} \approx 4.0 \text{ mm}^{-1} \text{ cm}^{-1}$). The extinctions are not the same because NADPH enhances the CT (22). Four eq of ferricyanide were required to restore the $E_{ox}$ spectrum, which is consistent with the assumption that borohydride produced the $E_{H4}$ form of TrxR and that two reducible disulfides occur in the enzyme. Curiously, the reoxidation from $E_{H2}$ to $E_{ox}$ by $[\text{Fe(CN)}_6]^{3-}$ was significantly slower ($0.013 \text{ s}^{-1}$) than that from $E_{H4}$ to $E_{H2}$ ($0.15 \text{ s}^{-1}$), so that several minutes were required after each ferricyanide addition for the spectrum to stabilize. Presumably, the conversion from $E_{H4}$ to $E_{H2}$ involves the reaction of $[\text{Fe(CN)}_6]^{3-}$ with the FADH⁻, because the equilibrium between $E_{H2}^A$ and $E_{H2}^B$ favors the latter overwhelmingly, the second phase is very slow.

Behavior of DmTrxR-1 toward Oxygen—The oxidation of $E_{H2}$ to $E_{ox}$ in the wild-type enzyme by molecular oxygen proceeds even more slowly than with ferricyanide. Freshly prepared DmTrxR-1 has a reddish color, and its spectrum indicates a partially reduced enzyme species ($E_{H2}$). When exposed to air, the color changes in a period of hours from reddish orange to yellow as reoxidation occurs. The high stability of $E_{H2}$ against auto-oxidation to $E_{ox}$ in vitro was a first hint that $E_{ox}$ does not serve as an intermediate in the catalysis under in vivo conditions.

The reactions of the three mutants of DmTrxR-1, C489S, C490S, and C489S/C490S, with oxygen are distinct from that of the wild-type enzyme. C490S and C489S/C490S are oxidized by O₂ within a few minutes, as are most members of this enzyme family (4). The C489S mutant, on the other hand, is more resistant to auto-oxidation than is wild-type DmTrxR-1, and thus it behaves very similarly to the closely related yeast glutathione reductase (4, 23). Even after 3 weeks of exposure to air, the enzyme stock solution had some reddish color, indicating partial reduction. Spectral analysis supported this assumption, the $\epsilon_{460 \text{ nm}}$ and $\epsilon_{540 \text{ nm}}$ values being approximately 9.8 and

Fig. 1. Reduction of DmTrxR-1 with NADPH. Panel A shows an anaerobic titration starting with $E_{ox}$ in 100 mM potassium phosphate buffer pH 7.0 (spectrum 1). Spectra 2–5 refer to 0.2, 0.4, 0.7, and 1.0 eq of NADPH (spectrum 5, dotted); 2 eq of NADPH are necessary for maximal 540 nm absorbance (spectrum 8, triangles). The inset shows the millimolar CT absorption at 540 nm as a function of NADPH eq added. Panel B shows the time course of reduction with 0.4 eq of NADPH followed by addition of 1 mM G6P and 0.2 unit G6PDH; spectrum 2 was taken immediately after mixing all components anaerobically, and spectra 3–9 were recorded after 6, 10, 14, 18, 23, 60, and 120 min, respectively.
The C489S and C490S mutants became turbid with time, requiring 5–6 weeks in the case of C489S and several days for C490S. The fine precipitate could not be cleared by filtration or reducing thioredoxin removed the cloudiness. This finding suggests the presence of a dithiol-disulfide redox center. This hypothesis is not only supported by the results from the NADPH and ferricyanide titrations described above, but also by the data from the C-terminal thiols of C490S can form an intermolecular disulfide via the remaining thiol, and these led to the turbidity. These observations suggest the specific functions of the C-terminal thiols (see below and "Discussion").

Accessible Thiol Groups in the Various Redox States of DmTrxR-1—Determination of the free thiol groups revealed further information about the redox state of the C-terminal cysteine pair. There are 9 cysteines per FAD in the protein, yet treatment of the oxidized enzyme ($E_{ox}$) with excess DTNB revealed less than 0.3 accessible thiol groups (Table I). This indicates that the 5 Cys residues not involved in redox-active disulfides are all buried or are otherwise inaccessible to DTNB. After reduction with borohydride of wild-type enzyme to the $E_{red}$ state, ~4 additional thiol groups are observed than are present in $E_{ox}$. Moreover, as expected, ~3 additional thiols are observed after reduction in C489S and ~2 additional in C489S/C490S. It is well known from other closely related flavoenzymes that the N-terminal dithiol pair, Cys489 and Cys490, whereas that on the right is completed by the C-terminal dithiol pair of the lower polypeptide chain, Cys489 and Cys490.

2.3 m$m^{-1}$ cm$^{-1}$, respectively, significantly different from the values observed for $E_{ox}$ species ($\varepsilon_{462}$ nm $\sim 11.9$ m$m^{-1}$ cm$^{-1}$ and $\varepsilon_{540}$ nm $\sim 0.5$ m$m^{-1}$ cm$^{-1}$ for wild-type DmTrxR-1). The observed stability of a form with CT absorbance may be the result of the C489S mutant form becoming trapped in a form resembling $E_{H2}$ (Schemes 2 and 3). The lack of Cys490 prevents formation of the $E_{H2}$ forms. The latter is probably required for reacting readily with O$_2$. This is evidence for Cys490 being the interchange thiol (see "Discussion").

After reduction of C490S with dithiothreitol and dialysis, the enzyme that has been stored aerobically for 3 weeks. It is well known from other closely related flavoenzymes that the N-terminal cysteine pair forms a disulfide as discussed in the previous section. The data further suggest that Cys489 in C490S and Cys430 in C489S are exposed to DTNB.

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**TABLE I**

| No. of accessible free thiol groups |
|------------------------------------|
| Native conditions                  |
| Denaturing conditions              |
| Oxidized enzyme                    |
| NaBH$_4$ treated enzyme            |
| Oxidized enzyme                    |
| NaBH$_4$ treated enzyme            |

|                     | Wild-type | C489S | C490S | C489S/C490S | 0.4 (0) | 2.3 (2) | 5.9 (5) | 7.4 (7) |
|---------------------|-----------|-------|-------|-------------|---------|---------|---------|---------|
| Oxidized enzyme     | 0.3 (0)   | 3.8 (4)| 5.7 (5)| 9.7 (9)     |         |         |         |         |
| NaBH$_4$ treated    | 3.1 (3)   | 6.7 (6)| 8.7 (6)| 9.5 (8)     |         |         |         |         |
| enzyme              | 0.6 (1)   | 2.1 (3)| 6.3 (6)| 7.8 (8)     |         |         |         |         |
| Wild-type           | 0.4 (0)   | 2.3 (2)| 5.9 (5)| 7.4 (7)     |         |         |         |         |

The evidence given above indicates that a second disulfide is present in $E_{ox}$. We conclude that it is made up of cysteines Cys489 and Cys490, which provide a catalytically significant dithiol-disulfide redox center. This hypothesis is not only supported by the results from the NADPH and ferricyanide titrations described above, but also by the data from the C-terminal mutants that are all lacking a functional redox center and are therefore inactive for Trx reduction.

Other Reducing Systems—Based upon the findings with NADPH, we applied alternative reduction methods to study the $E_{H4}$ species in which the FAD and both redox-active Cys pairs are reduced. Reduction with the xanthine-xanthine oxidase system (19) gave incomplete formation of $E_{H4}$ (data not shown). We had similar difficulties in forming $E_{H4}$ when applying the photoreduction method in the presence of EDTA (20). With 0.12 eq of 5-deazaflavin sulfonate present, CT absorbance was not observed (Fig. 2, curves 1–5), rather the spectral characteristics indicate the formation of an anionic FAD semiquinone species (24). Subsequent addition of 0.1 eq of

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**SCHEME 1** Model of the dimeric structure of DmTrxR-1. The dimer interface is shown as a diagonal line with a circle at the center. Amino acid residues from the two polypeptide chains are distinguished by the absence or presence of primes. The NADPH binding site, flavin, N-terminal dithiol pair, and C-terminal dithiol pair on the right constitute one catalytic unit, whereas those on the left form the other unit. However, the active site on the left is completed by the C-terminal dithiol of the upper polypeptide chain, Cys489 and Cys490, whereas that on the right is completed by the C-terminal dithiol pair of the lower polypeptide chain, Cys489 and Cys490.

**SCHEME 2** Model of the catalytic mechanism of DmTrxR-1. The reductive half-reaction involves the steps designated by rate constants $k_1\rightarrow k_4$, and the oxidative half-reaction the steps $k_5\rightarrow k_7$. NADPH activates the enzyme in the steps designated $k_1\rightarrow k_5$, and the catalytic cycle is $k_7\rightarrow k_{ox}$. MC, Michaelis complex.
MV promoted electron equilibration to yield a spectrum best explained by a mixture of $E_H$ and a small amount of the semiquinone (spectrum 6). Increasing the MV concentration to be equimolar with DmTrxR-1, and further irradiation gave a final spectrum indicating no detectable semiquinone and almost full reduction to $E_H$ (spectrum 7). Spectra taken immediately after irradiation revealed low MV radical concentrations that gradually decreased below detection level within 5–10 min. This indicates that the redox potential of the flavin is similar to that of the MV/MV$^-$ pair ($E_0 = \sim -400$ mV at pH 7.0) (25); this is extremely low relative to other members of the enzyme family (4). These data, knowledge of the redox milieu in eukaryotic cells (−200 to −250 mV) (26, 27), and the considerable instability of $E_H$ led to the conclusion that the 6-electron reduced form of DmTrxR-1 is not relevant in vivo. The low redox potential also implies that FADH$^-$ is not bound to DmTrxR-1 as tightly as is FAD.

The Role of Cys$^{490}$ in Electron Transfer in DmTrxR-1—It was mentioned above that the redox behavior of the C489S mutant form of DmTrxR-1 is distinct from the other two mutants. We assumed that the extremely persistent CT absorbance resulted because the protein was trapped as a disulfide between the single C-terminal Cys$^{490}$ and Cys$^{62}$ (Scheme 3). Thus, C489S should become readily auto-oxidizable (like C490S or C489S/C490S) upon covalent modification of Cys$^{490}$. DmTrxR-1 C489S was therefore incubated with a 100-fold excess of iodoacetamide, and the spectral changes were followed. Within 1.5 h the initial spectrum changed to the typical $E_{ox}$ spectrum. After removing free iodoacetamide from the solution, we incubated the enzyme with 1 eq of NADPH aerobically, resulting in a spectrum typical of reduced enzyme ($E_L$ as in Fig. 1A, spectrum 5). In the presence of oxygen, the alkylated enzyme reoxidizes to $E_{ox}$ when the NADPH is fully consumed, whereas unmodified C489S is not auto-oxidized, and retains the CT absorbance.

The Reductive Half-reaction with NADPH—Reduction of DmTrxR-1 with NADPH resulted in patterns of absorbance change that are typical for flavoenzymes like mammalian or plasmodial high $M_r$ TrxR (3) as shown in the left panel of Fig. 3. The patterns are somewhat more complex than those observed for glutathione reductase, an enzyme having only an N-terminal redox-active disulfide (16). Spectra recorded in the first −30 ms show formation of a broad band centered at 680 nm that is indicative of an FADH$^-$–NADPH$^+$ charge-transfer complex (spectra 3 and 4). Simultaneously, the 440–460 nm region shows flavin reduction. Subsequent spectra (6–8) show some reoxidation of the flavin at 440–460 nm. The concomitant reduction of the disulfide to form the thiolate-flavin charge-transfer complex (spectrum 8) shows up as a “triangular” motif between 505 and 580 nm when the absorbance at 540 nm becomes maximal.

The inset to Fig. 3 (left panel) shows the time dependence of the changes at 360, 440, 505, and 540 nm upon mixing with 4 eq of NADPH; the traces at 440 and 505 nm decreased, showing flavin reduction in the first 20 ms. From −30–200 ms the absorbance at 440 and 505 nm increased, indicating flavin reoxidation, and then remained almost constant. The charge-transfer absorbance at 540 nm increased to −50% of its maximal value in the first 30 ms, and reached a maximum at −200 ms. The traces were best fitted with eigenvalues (apparent rate constants) of 95–120, 49, and 21 s$^{-1}$. The slow rate (21 s$^{-1}$) is not associated with a single process but reflects dithiol-disulfide interchange as well as the reaction with the second molecule of NADPH. The change at 680 nm (FADH$^-$–NADPH$^+$ charge-transfer complex formation and decay) is most clearly observed in the experiment with 1.7 eq of NADPH, as shown in the inset.

The rate of the fastest phase was almost the same with either 30 μM (1.7 eq) or 56 μM (3.3 eq) NADPH. This implies that the $K_D$ for NADPH is considerably less than 30 μM. The first equivalent of NADPH, as calculated from the 360 nm absorbance, is consumed in the fast phase that is associated with flavin reduction. Further NADPH reacted in subsequent steps out to −200 ms. Consumption of 2 eq of NADPH was also observed in the static titration. In both cases, the flavin remains mostly oxidized, so that the results are consistent with the enzyme containing two intramolecular redox centers in addition to the flavin.

When the reaction of DmTrxR-1 with 1 eq of NADPH was compared with reactions using 2, 3, and 5 eq, a third phase could be more clearly resolved, as shown in Fig. 3 (right panel). This shows the spectra at the end of each reaction; as expected, the spectrum for 1 eq (spectrum 2) indicates that the flavin is more oxidized (more absorbance near 462 nm and less charge-transfer complex as indicated at 540 nm). The single wavelength kinetic data (inset) show flavin reduction as a decrease...
in absorbance at 462 nm in the first phase (−95–120 s⁻¹) for both 1 and 5 eq, but the subsequent phases differ. The second phase (49 s⁻¹) is clearly shown at 540 nm, where there is an increase in absorbance caused by formation of the CT complex. At 462 nm, the formation of oxidized flavin is denoted by a small increase in absorbance at the same rate. The third phase for 1 eq shows a small decrease at 462 nm and an additional increase at 540 nm, whereas, with 5 eq, both wavelengths show increases in absorbance in this phase. These differences may reflect the binding of NADPH to the E_H4 form when 5 eq are present as well as other reductive steps that would mask the results with greater concentrations.

Excess NADPH led to partial further reduction of the E_H4 form (data not shown). However, this was a very slow process that required minutes for completion. Thus, not only the thermodynamic aspects that were described above, but also the kinetic data supported the conclusion that E_HC can be clearly excluded from playing an active role in catalysis.

Reduction of the C-terminal Mutants C489S, C490S, and C489S/C490S by NADPH—The spectral changes of the three C-terminal mutants were very similar to the ones observed in wild-type DmTrxR. Rates of 150, 42, and 0.4 s⁻¹ described the data quite well. Importantly, however, the rate at 21 s⁻¹ observed with wild-type enzyme and attributed to dithiol-disulfide interchange, as well as to reaction of the second molecule of NADPH, was not seen with the mutant forms. In all cases there was consumption of only 1.2 eq of NADPH, and this occurred in the fast phases. Thus, an E_H2 form was the product of the reaction. We concluded that all these mutants lack a functional redox center in their C termini. The fact that none of the mutants were capable of reducing the natural substrate thioredoxin is consistent with this conclusion.

Reaction of DmTrxR-1 at the E_H4 Level with DmTrx-2—DmTrxR-1 was reduced with NaBH₄ and mixed in the stopped-flow spectrophotometer with oxidized DmTrx-2. Fig. 4 shows the spectrum of E_H4 and the spectrum after reaction with 4 eq of DmTrx-2. The spectrum of E_HC is shown for comparison. Clearly, equilibrium was established and the enzyme did not completely reoxidize. Upon reaction with excess DmTrx-2, the absorbance at 540 nm decreased from −3.3 mM⁻¹ cm⁻¹ to −1.7 mM⁻¹ cm⁻¹ in the first 20 ms (and finally to −1.2 mM⁻¹ cm⁻¹), and the absorbance at 460 nm increased from −9.2 mM⁻¹ cm⁻¹ to −10.6 mM⁻¹ cm⁻¹ (and finally to −11.2 mM⁻¹ cm⁻¹). The values after 20 ms indicate that the enzyme had reoxidized to the E_HC level. The further reoxidation detected between 20 ms and 10 s represented the partial reoxidation of E_HC to E_OX. The final values of ε_{540 nm} ~ 1.3 mM⁻¹ cm⁻¹ and ε_{460 nm} ~ 11.3 mM⁻¹ cm⁻¹ were higher and lower, respectively, than those for E_OX; thus, even an excess of 4 eq of DmTrx-2 did not fully reoxidize the enzyme. This slow kinetic behavior of E_HC with DmTrx-2 provided evidence that the E_OX enzyme species is not involved in catalysis. The enzyme therefore cycles between E_H4 and E_HC during catalysis.

DTNB as a Substrate of Wild-type DmTrxR-1—When TrxR-1 was reduced to E_H4 with NaBH₄ and reacted with DTNB in the rapid reaction spectrophotometer, complete reoxidation of the enzyme was observed. The kinetic trace at 412 nm showed the release of 3.7 eq of TNB anion (ε_{412 nm/TNB anion} = 13.6 mM⁻¹ cm⁻¹); thus, nearly 2 molecules of DTNB were reduced by E_HC. A fast and a slower phase were observed in the DTNB reduction process. The rate of the reaction was second order in DTNB, indicating that the Kₖᵣ for DTNB is greater than 1 mM.
The disulfide adjacent to the flavin is referred to as the N-terminal disulfide to distinguish it from the group at the C terminus. As in the case of high $M_r$ TrxR from mammals or the malaria parasite, it is the C-terminal redox center that is responsible for the electron transfer to the substrate Trx. This organization of the active site is reminiscent of mercuric ion reductase, but there the C-terminal dithiol of the “other” subunit provides mercuric ion ligands rather than a catalytic group as in TrxR (28).

The Model of the Catalytic Cycle of DmTrxR-1—Before discussing the data, we will introduce our model for activation of DmTrxR-1 and catalysis (Scheme 2). The oxidized enzyme is shown at the top with Cys$^{57}$ and Cys$^{62}$ adjacent to the flavin and Cys$^{490}$ and Cys$^{490}$ near the C terminus present as disulfides. Because peptide bonds are normally planar, one predicts that a disulfide between adjacent cysteines will be strained. However, the peptide bond can be twisted slightly to relieve the strain (29). In the case of the TrxR mutant Sec$^{498} \rightarrow$ Cys from rat (RrTrxR U498C) (6), it was argued that the low reactivity of this mutant was the result of the difficult formation of the disulfide between the cysteines that are homologous to Cys$^{469}$ and Cys$^{490}$ in DmTrxR-1. However, the structure of DmTrxR-1 would indicate that this is not the case. The main difference between the RrTrxR U498C and wild-type DmTrxR are the amino acid residues adjacent to the cysteines; there is a Ser-Cys-Cys-Ser sequence in Dro sophila, whereas a Gly-Cys-Cys-Gly motif is found in the rat enzyme mutant. The high catalytic activity of DmTrxR-1 indicates that the serine residues make important contributions to the reactivity of the C-terminal disulfide; data elaborating this point will be published elsewhere.

The first equivalent of NADPH forms a Michaelis complex (MC$_1$) that leads to an equilibrium mixture of the four 2-electron reduced species, with EH$_2^+$, the species having high thiolate-flavin charge transfer, predominating. It is assumed that EH$_2^+$ and EH$_2^-$ have similar spectral properties and that EH$_2^+$ has spectral properties similar to $E_{ox}^-$. Addition of a second

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**Fig. 3. Spectra of wild-type DmTrxR-1 during the reductive half-reaction.** Left panel, wild-type DmTrxR-1 in 100 mM potassium phosphate buffer, pH 7.0, was reacted with 4 eq of NADPH at 10 °C. Spectra shown were recorded at time 0, 1.5 ms, 4 ms, 10 ms, 29 ms, 49 ms, 100 ms, and 1.425 s. The inset shows the time dependence of the traces at 360, 440, 505, and 540 nm after mixing the enzyme with 4 eq of NADPH, as well as a trace at 680 nm obtained from an experiment with only 1.7 eq of NADPH. Full-scale delta absorbance was 0.1 at 360 nm, 0.025 at 440 nm, 0.02 at 505 nm, 0.05 at 540 nm, and 0.015 at 680 nm. Right panel, wild-type DmTrxR-1 in 100 mM potassium phosphate buffer, pH 7.0, was reacted with 0, 1, 2, 3, and 5 eq of NADPH at 10 °C. The inset shows the time dependence of the traces at 462 and 540 nm for 1 and 5 eq. Full-scale delta absorbance was 0.08.
equivalent of NADPH leads, via MC2, to EH4\textsuperscript{A} and EH4\textsuperscript{B}. This completes what is referred to as the reductive half reaction. EH4\textsuperscript{B} reacts with Trx\textsubscript{S2} to begin the oxidative half reaction with formation of a mixed disulfide between Trx and the enzyme C-terminal thiols; this is the first step of dithiol-disulfide interchange that is completed with the release of reduced Trx(SH)\textsubscript{2}, leaving DmTrxR in the EH2\textsuperscript{B} state ready for another round of catalysis. The dissociation steps of NADP\textsuperscript{+} are not specified.

Reduction of Wild-type DmTrxR-1 by NADPH—The titration experiment shown in Fig. 1A indicated that DmTrxR-1 has two disulfide redox centers. The first equivalent of NADPH yielded a high proportion of the total thiolate-flavin CT, indicating that the product was primarily EH2\textsuperscript{B} (Scheme 2). The second equivalent of NADPH gives EH4\textsuperscript{B}, which has slightly more intense CT absorbance. From the decrease in absorbance near 450 and 540 nm shown in spectrum 8, it can be concluded that some reduced flavin has formed upon addition of excess NADPH (inset). The finding that very little EH4\textsuperscript{A} is formed, even with excess NADPH present, indicates that the redox potential of FAD in EH4\textsuperscript{A} is very low. Reduction of the enzyme with a stronger reducing system, G6P and G6PDH, is shown in Fig. 1B. The initial product is primarily EH4\textsuperscript{B} (spectrum 2), and complete reduction yields EH6\textsuperscript{B} (spectrum 9). Alternate methods of reduction confirmed that the redox potential of the flavin was perhaps as low as \(-400\) mV, unusually low even for this enzyme family. The comparable redox potential for lipoamide dehydrogenase is \(-346\) mV (4).

The Reductive Half-reaction with NADPH—The rapid reaction experiments of wild-type DmTrxR-1 with NADPH permitted the observation of intermediates in the reaction (Fig. 3, left panel). Hydride transfer from the first NADPH to the flavin is observed as the formation, at a rate of \(-95-120\) s\textsuperscript{-1} (within the first 30 ms), of a broad spectral band centered at 680 nm; in this charge-transfer complex, reduced flavin is the donor and NADP\textsuperscript{+} is the acceptor (22). With some flavoprotein disulfide reductases, this cannot be observed because the subsequent formation of the thiolate-flavin charge-transfer species is too fast. With DmTrxR-1, flavin reoxidation can be identified as a separate step at \(-49\) s\textsuperscript{-1}. In this reaction, the absorbance at 680 nm decreases, whereas that at 540 nm increases. The simultaneous flavin reoxidation was best detected as an increase at 440 or 505 nm after 30 ms (k2 \(-49\) s\textsuperscript{-1}). It is suggested that the slower phase that follows (k3 \(-21\) s\textsuperscript{-1}, as seen by a slight increase in absorbance at 460 and 505 nm) is a result of electron transfer to the C-terminal redox pair, but these observations are complicated by spectral changes caused by reaction of the second molecule of NADPH. Absorbance changes at 360 nm, which is an isosbestic point between Eox and EH4\textsuperscript{B}, show that 2 eq of NADPH have reacted by 200 ms; thus, the product of the reductive half reaction is primarily EH4\textsuperscript{B}. “Over-reduction” to EH6\textsuperscript{B} is not observed on this time scale, indicating that it is neither thermodynamically nor kinetically relevant for catalysis. Referring to Scheme 2, we ascribe the observed rates of \(-95-120\) and \(-49\) s\textsuperscript{-1}, observed in the reductive half reaction, to k3 and k5, respectively, and the rate of 21 s\textsuperscript{-1} to some combination of k5 and k12 as the various forms of EH6 equilibrate and NADPH displaces NADP\textsuperscript{+}.

The reductive half-reaction was much simpler with the C-terminal mutants, C489S, C490S, and C489S/C490S, because only 1 eq of NADPH is required to reduce these enzyme forms. Reduction was observed as two fast phases, with rates very similar to those found with wild-type enzyme. The third phase observed with wild-type enzyme (21 s\textsuperscript{-1}) is essentially absent in the reactions with the mutant forms of enzyme; this is consistent with the assignment of the third phase to the disulfide interchange with the C-terminal Cys-pair in wild-type enzyme.

Reaction of DmTrxR-1 EH4 with DmTrx-2—The reaction of DmTrx-2 with enzyme reduced by borohydride (EH4\textsuperscript{B}) (Fig. 4 and Scheme 2) takes place in two steps, namely formation and breakdown of the mixed disulfide between the enzyme and Trx. This is the final step of the catalytic cycle, and the enzyme is left as an equilibrium mixture of EH4\textsuperscript{A} forms. Excess thiore-

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**Fig. 4.** Wild-type DmTrxR-1 in the oxidative half-reaction. Wild-type DmTrxR-1 reduced with a 100-fold excess of NaBH4 (spectrum 1) was reacted with 4 eq of DmTrx-2 (spectrum 2). The E\textsubscript{ox} spectrum of DmTrxR-1 is given as a reference. The inset shows the kinetic traces at 460 and 540 nm associated with the oxidative half-reaction. The traces could be fitted with rate constants of \(-140, 11,\) and \(0.5\) s\textsuperscript{-1}. The rapid reaction experiments are shown in Fig. 3. The kinetic traces at 460 and 540 nm, as well as absorbance at 680 nm, are shown in spectrum 8. The titration experiment shown in Fig. 1A indicated that DmTrxR-1 has two disulfide redox centers. The first equivalent of NADPH yielded a high proportion of the total thiolate-flavin CT, indicating that the product was primarily EH2\textsuperscript{B} (Scheme 2). The second equivalent of NADPH gives EH4\textsuperscript{B}, which has slightly more intense CT absorbance. From the decrease in absorbance near 450 and 540 nm shown in spectrum 8, it can be concluded that some reduced flavin has formed upon addition of excess NADPH (inset). The finding that very little EH4\textsuperscript{A} is formed, even with excess NADPH present, indicates that the redox potential of FAD in EH4\textsuperscript{A} is very low. Reduction of the enzyme with a stronger reducing system, G6P and G6PDH, is shown in Fig. 1B. The initial product is primarily EH4\textsuperscript{B} (spectrum 2), and complete reduction yields EH6\textsuperscript{B} (spectrum 9). Alternate methods of reduction confirmed that the redox potential of the flavin was perhaps as low as \(-400\) mV, unusually low even for this enzyme family. The comparable redox potential for lipoamide dehydrogenase is \(-346\) mV (4).

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Thioredoxin Reductase from D. melanogaster

doxin did not fully oxidize the enzyme to $E_{ox}$. This observation leads to the conclusion that $E_{H_2}$ and $E_{ox}$ are the only species involved in catalysis. We suggest that the rate of $-140 \text{ s}^{-1}$ is associated with $k_1$ and that this reaction leads to protonation of the thiolate of Cys62, the charge transfer donor; this step is associated with loss of $\Delta G^\circ$ of the CT absorbance. There is an important caveat; not shown in this scheme is the active site base, His64, positioned so that it probably facilitates proton transfer among all four thiols. The rate of 11 s$^{-1}$ may be associated with steps from $E_{H_2}^D$ to $E_{H_2}^P$ that would lead to further loss of charge transfer. This rate of 11 s$^{-1}$ is clearly important in determining the overall rate of catalysis, which is $-5 \text{ s}^{-1}$ at 10°C. An alternative mechanism would involve the reaction of $E_{H_2}^P$ with Trx (not shown in Scheme 2). However, we consider this unlikely.

In contrast to thioredoxin, DTNB or ferricyanide are capable of fully reoxidizing wild-type DmTrxR-1 that had been reduced with borohydride. The reaction requires 2 eq of DTNB, or 4 eq of ferricyanide, confirming that borohydride-reduced enzyme is an $E_{H_2}$ species. DTNB also reoxidizes all C-terminal mutants. DmTrxR is a substrate of the mutants, but the $K_m$ values for DTNB with C489S, C490S, and C489S/C490S are at least 7-fold higher than that for the wild-type enzyme ($K_m \sim 700 \mu M$). This indicates that the small disulfide compound DTNB, unlike the 12-kDa protein thioredoxin, is able to react directly with the N-terminal cysteine pair. Wild-type enzyme reacts with DTNB preferentially via the C-terminal redox center.

The Role of Cys$^{490}$ in Dithiol-Disulfide Interchange in DmTrxR-1—The unusual auto-oxidation behavior of the C489S mutant suggests which of the C-terminal cysteine residues is responsible for the interchange with Cys$^{57}$ in catalysis (Scheme 3). The remaining thiol of Cys$^{490}$ is able to attack Cys$^{57}$. This results in an enzyme species with significant CT absorbance, species A. The C490S mutant does not show similar behavior, suggesting that the remaining thiol of Cys$^{490}$ does not normally interchange with Cys$^{57}$. By covalently modifying the enzyme (Km C490S are at least 7-fold higher than that for the wild-type enzyme ($K_m \sim 700 \mu M$). This indicates that the small disulfide compound DTNB, unlike the 12-kDa protein thioredoxin, is able to react directly with the N-terminal cysteine pair. Wild-type enzyme reacts with DTNB preferentially via the C-terminal redox center.

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