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Evidence for two conformational states of thioredoxin reductase from *Escherichia coli*: Use of intrinsic and extrinsic quenchers of flavin fluorescence as probes to observe domain rotation

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Abstract

Thioredoxin reductase (TrxR) from *Escherichia coli* consists of two globular domains connected by a two-stranded β sheet: an FAD domain and a pyridine nucleotide binding domain. The latter domain contains the redox-active disulfide composed of Cys 135 and Cys 138. TrxR is proposed to undergo a conformational change whereby the two domains rotate 66° relative to each other (Waksman G, Krishna TSR, Williams CH Jr, Kuriyan J, 1994, *J Mol Biol 236*:800–816), placing either the redox active disulfide (FO conformation) or the NADPH binding site (FR conformation) adjacent to the flavin. This domain rotation model was investigated by using a Cys 138 Ser active-site mutant. The flavin fluorescence of this mutant is only 7% that of wild-type TrxR, presumably due to the proximity of Ser 138 to the flavin in the FO conformation. Reaction of the remaining active-site thiol, Cys 135, with phenylmercuric acetate (PMA) causes a 9.5-fold increase in fluorescence. Titration of the PMA-treated mutant with the nonreducing NADP(H) analogue, 3-aminopyridine adenine dinucleotide phosphate ($AADP^+$), results in significant quenching of the flavin fluorescence, which demonstrates binding adjacent to the FAD, as predicted for the FR conformation. Wild-type TrxR, with or without PMA treatment, shows similar quenching by $AADP^+$, indicating that it exists mostly in the FR conformer. These findings, along with increased EndoGluC protease susceptibility of PMA-treated enzymes, agree with the model that the FO and FR conformations are in equilibrium. PMA treatment, because of steric limitations of the phenylmercuric adduct in the FO form, forces the equilibrium to the FR conformer, where $AADP^+$ binding can cause fluorescence quenching and conformational restriction favors proteolytic susceptibility.

Keywords: conformation; Cys modification; FAD fluorescence; flavoproteins; fluorescence quenching; limited proteolysis; thioredoxin reductase

The thioredoxin reductase from *Escherichia coli* catalyzes the transfer of reducing equivalents from NADPH to thioredoxin, which subsequently performs many important cellular roles. The substrate thioredoxin is a small (M_r = 11,700) protein that contains a single redox-active disulfide and is involved in ribonucleotide reduction (Thelander, 1967; Holmgren, 1989), bacteriophage assembly (Russel & Model, 1986), and protein folding (Yasukawa et al., 1995). Thioredoxin reductase is a member of a class of related flavoenzymes containing a redox-active disulfide, which includes lipoamide dehydrogenase and glutathione reductase (Williams, 1992, 1995). Thioredoxin reductase is a dimer that contains one FAD and one redox-active disulfide per monomer. The flow of electrons is from NADPH to FAD, from reduced FAD to the active-site disulfide, and from the active-site dithiol to thioredoxin via dithiol–disulfide interchange.

The crystal structure reveals that each monomer consists of two globular domains connected by a double-stranded β sheet. One domain contains the FAD binding site, whereas the other domain has the NADPH binding site and the redox-active disulfide (Kuriyan et al., 1991; Waksman et al., 1994). A significant finding in the structure is that there is no obvious path for the flow of electrons from NADPH via the flavin ring to the active-site disulfide as there

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Abbreviations: TrxR, thioredoxin reductase; $TrxR^{135S}_{138S}$, wild-type thioredoxin reductase with the redox active cysteines, Cys 135 and Cys 138 present as the disulfide; $TrxR^{135SH}_{138OH}$, thioredoxin reductase with Cys 138 mutated to a serine; $TrxR^{135OH}_{138SH}$, thioredoxin reductase with Cys 135 mutated to a serine; $TrxR^{135S\text{-}PMA}_{138OH}$, $TrxR^{135SH}_{138OH}$ treated with phenylmercuric acetate; $TrxR^{135S\text{-}MMTS}_{138OH}$, $TrxR^{135SH}_{138OH}$ treated with methylmethane thiosulfonate; $TrxR^{135S\text{-}PMA}_{138S\text{-}PMA}$, wild-type TrxR reduced with NADPH and treated with phenylmercuric acetate and reoxidized; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, 1,4-dithiothreitol; MMTS, methyl methane-thiosulfonate; PMA, phenylmercuric acetate; $AADP^+$, 3-aminopyridine adenine dinucleotide phosphate. We have taken our symbolism for enzyme mutants from the work of T. Creighton.

is for other members of this enzyme family. The active-site disulfide is adjacent to the flavin and buried such that it cannot interact with the protein substrate thioredoxin, and the nicotinamide ring of bound NADPH is located 17 Å away from the flavin ring.

The lack of a direct pathway for reducing equivalents from NADPH to the active-site disulfide led to a proposal that the enzyme undergoes a large conformational change during catalysis whereby the two domains rotate 66° relative to each other (Waksman et al., 1994). This rotation and counter-rotation alternately places either the nicotinamide ring of NADPH or the active-site disulfide adjacent to the flavin and allows for the active-site dithiol to move from a buried position to the surface, where it would reasonably react with the protein substrate thioredoxin. Thus, there may be two conformational states for thioredoxin reductase: the form in which the active-site disulfide is close to and can oxidize the flavin during catalysis (FO), and the form in which the nascent dithiol is exposed to solvent and the pyridine nucleotide binding site is close to and can reduce the flavin (FR). These are diagrammed in Figure 1A for the $TrxR|^{135SH}_{138OH}$ mutant form of TrxR. Figure 1B shows the observed FO conformation (top) and the putative FR conformation (bottom) in stereo format for the C138S mutant form of TrxR. The FO form is favored in the packed crystal, but in solution, we assume that there is an equilibrium between

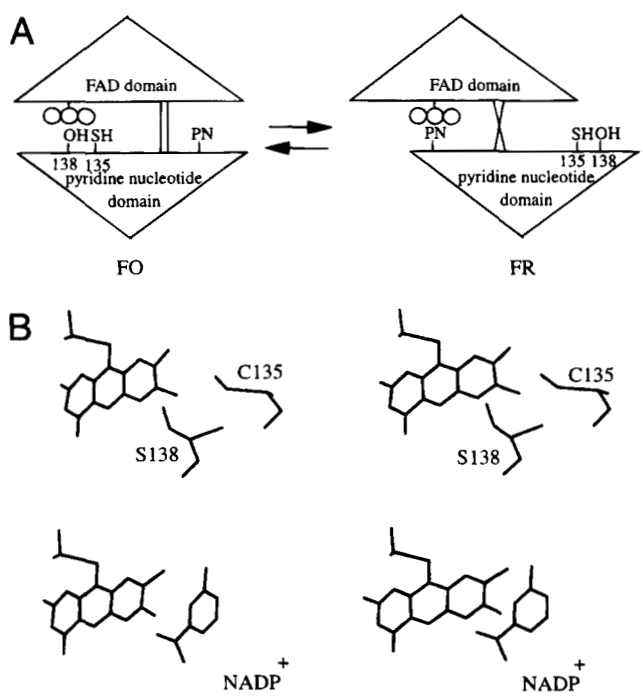

Fig. 1. Representations of the C138S mutant of TrxR in the FO and postulated FR conformers. **A:** Diagram of the two forms. The FAD and pyridine nucleotide domains are indicated and connected by lines depicting the double-stranded β-sheet. The three circles represent the FAD, and PN indicates bound pyridine nucleotide. Although the difference between the FO and FR is shown as a 180° flip in this illustration, it is postulated actually to be 66°. **B:** Stereo view of the *re* face of the FAD in the FO and FR conformers showing the FO form on the top and FR on the bottom. Data for the FO form are from the crystal structure (Kuriyan et al., 1991; Waksman et al., 1994) (Brookhaven Protein Data Bank entry 1tdf) and the FR form is from the model (Waksman et al., 1994) with $NADP^+$ bound. The closest distances from the FAD are 3.1 Å (FAD C4a to $NADP^+$ O6) and 3.1 Å (FAD C4a to Ser 138 O). Stereo view was generated from the SYBYL software package (Tripos Associates, St. Louis, Missouri).

these two forms necessary for catalysis; data consistent with that assumption have been presented (Lennon & Williams, 1997; Williams, 1995).

The TrxR redox active disulfide consists of Cys 135 and Cys 138. One active-site mutant, $TrxR|^{135OH}_{138SH}$, was used to construct a stable, covalent complex with the substrate thioredoxin that also had one of its active-site cysteines mutated to a serine. The two proteins were linked through a disulfide bond between their respective remaining single cysteine active-site residues (Wang et al., 1996). Based on the proposed FO/FR model, this cross-linked complex should restrict the enzyme to the FR conformation because this is the only form in which the active-site thiol is exposed to the protein surface and can react with the large mutant thioredoxin substrate. Data from reductive titrations, charge transfer absorbance formation, and enzymatic activities were consistent with the $TrxR|^{135OH}_{138SH}$ in the complex being restricted to the FR conformation (Wang et al., 1996).

Another mutated form of TrxR in which the other active-site cysteine was changed to a serine, $TrxR|^{135SH}_{138OH}$ (Prongay et al., 1989), has been reevaluated recently (Veine & Williams, 1997). One important characteristic of this mutant is that its flavin fluorescence is only 7% that of the wild-type enzyme. The quenching of the fluorescence is postulated to be due to the proximity of Ser 138 to the flavin ring, which occurs only in the FO conformation (Fig. 1) (Mulrooney & Williams, 1997). Here we use the intrinsic quenching of flavin fluorescence of this mutant to examine the conformational state of TrxR and provide evidence that supports the existence of both the FO and FR forms in solution. Limited proteolysis of the mutant and wild-type TrxR also provides evidence that an equilibrium exists between the two conformational states.

Results

Spectral changes of $TrxR|^{135SH}_{138OH}$ upon reaction with PMA

The $TrxR|^{135SH}_{138OH}$ mutant is unique among TrxR altered forms that have been examined in that it has very low fluorescence and the absorbance peaks are blue-shifted at 368 nm and 448 nm relative to wild-type enzyme; the 368/448 nm absorbance ratio is 0.83. The fluorescence excitation peaks differ from the absorbance peaks and are found at 380 nm and 456 nm (cf. Figs. 2A, 3). If the FO and FR conformations are in an equilibrium, as depicted in Figure 1A, then the low fluorescence and unique absorbance spectrum of $TrxR|^{135SH}_{138OH}$ may indicate that the equilibrium is mostly toward the FO side, where Cys 135 is close to the FAD in a tightly packed space. If $TrxR|^{135SH}_{138OH}$ was reacted with a thiol-specific reagent such as PMA, then a phenylmercuric adduct would form presumably on the remaining active center thiol Cys 135. Space restrictions in the FO form would dictate that only those molecules in the FR conformation would react. Thus, reaction with PMA would shift the equilibrium mostly to the FR conformation and relieve the fluorescence quenching. To test this, PMA was titrated into $TrxR|^{135SH}_{138OH}$ and fluorescence spectra were recorded at each addition. The titration resulted in a 9.5-fold increase in fluorescence (Fig. 2A). The reaction is virtually stoichiometric with an end point of 1.1 equivalents (Fig. 2B). There are also pronounced absorbance changes upon addition of PMA with the main peak shifting from 448 nm to 455 nm and the 368 nm peak shifting to 378 nm with an accompanying increase in absorbance to give a 378/455 nm ratio of 0.95 (Fig. 3). The absorbance peaks following

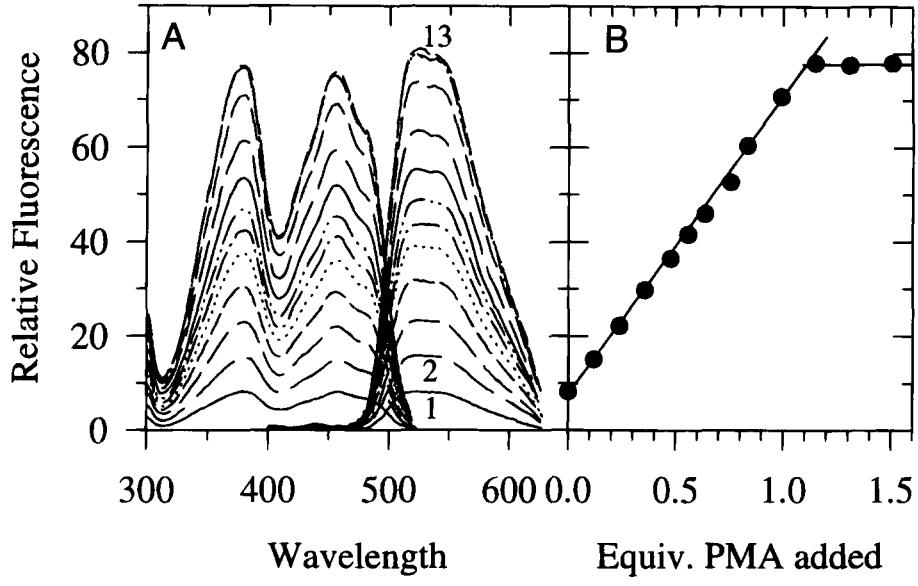

Fig. 2. Fluorescence changes resulting from PMA treatment of $TrxR^{135SH}_{138OH}$. **A:** Increase in fluorescence excitation (left spectra, 540 nm emission) and emission (right spectra, 380 nm excitation) during titration of $TrxR^{135SH}_{138OH}$ with PMA. Spectra (1–13) were taken at PMA equivalents of 0, 0.12, 0.24, 0.36, 0.48, 0.56, 0.64, 0.75, 0.83, 0.99, 1.15, 1.31, 1.51. **B:** Fluorescence changes upon titration of $TrxR^{135SH}_{138OH}$ with PMA measured at 456 nm excitation and 540 nm emission.

PMA treatment are very close to those observed in the excitation spectra.

$TrxR^{135S}_{138S}$ reduced with excess NADPH, reacted with PMA on both thiols, and reoxidized has virtually the same fluorescence as the untreated enzyme because both are presumably in the FR conformation. Wherever the fluorescence of two enzyme forms is compared in order to assess the conformation, FO or FR, the flavin must be in the oxidized state because reduced FAD in TrxR has very low fluorescence.

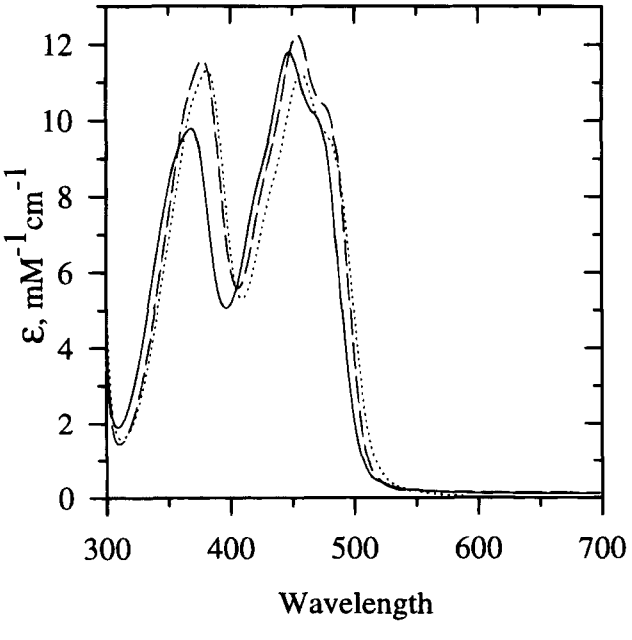

Fig. 3. Spectra of $TrxR^{135SH}_{138OH}$ before (———) and after (— — —) addition of three equivalents of PMA. For comparative purposes, the spectrum of $TrxR^{135S}_{138S}$ is also shown (·····).

Stopped-flow fluorescence measurements of the reaction of PMA with $TrxR^{135SH}_{138OH}$

The reaction of a thiol with a mercurial is slow relative to the rates of even the slowest steps in catalysis by TrxR (Evans & Lipscomb, 1979). As expected, the rate of the reaction of PMA with $TrxR^{135SH}_{138OH}$ was measured and found to be too slow to allow an estimate of the rate of rotation. The fluorescence increase was fit to a single exponential and the resulting rates were found to range from 0.08 s^{-1} to 0.98 s^{-1}. The rates are roughly proportional to the PMA concentration and the second-order rate constant is 6,160 M^{-1} s^{-1} (data not shown). The rate of the slowest step in catalysis, thought to be rotation, is 65 s^{-1} (Lennon & Williams, 1997).

Spectral changes upon reaction of MMTS with $TrxR^{135SH}_{138OH}$

From the above results, it was logical to test a similar strategy using a thiol-specific reagent that gave a smaller adduct, such as MMTS. A single addition of 1.2 equivalents of MMTS to $TrxR^{135SH}_{138OH}$ causes an 8.1-fold increase in the fluorescence when measured at the 456 nm excitation peak, as well as absorbance changes almost identical to those observed for PMA treatment. If the MMTS reaction with Cys 135 is truly causing an equilibrium shift toward the FR conformation, then removal of the thiomethyl adduct should return the $TrxR^{135SH}_{138OH}$ back to near its original spectral properties. Subsequent reaction of $TrxR^{135S\text{-}MMTS}_{138OH}$ with 30 equivalents of DTT at 25 °C to reduce Cys 135 back to the thiol form resulted in very slow spectral changes. After allowing the DTT to react overnight at 4 °C, the fluorescence had decreased 6.5-fold and the absorbance spectrum was identical to the starting spectrum before MMTS treatment (data not shown). Addition of three equivalents of MMTS in a separate experiment resulted in an

8.5-fold increase in fluorescence excitation and an identical change in absorbance as seen for 1.2 equivalents.

AADP$^+$ titrations

AADP$^+$ is an NADP(H) analogue that shares similar spectral and fluorescence properties (Tu, 1981), but is in the oxidized state and thus will not reduce TrxR. If AADP$^+$ binds to TrxR in the FR conformation in a similar way that NADPH is postulated to bind (see Fig. 1B, lower), then significant spectral changes and fluorescence quenching would be expected to occur. Titration of AADP$^+$ into $TrxR^{135S\text{-}PMA}_{138OH}$ or $TrxR^{135S\text{-}MMTS}_{138OH}$ resulted in profound fluorescence quenching. An example of AADP$^+$ titration into $TrxR^{135S\text{-}PMA}_{138OH}$ is shown in Figure 4A, and the concomitant absorbance changes are shown in Figure 4B. Addition of AADP$^+$ to $TrxR^{135SH}_{138OH}$ results in a small decrease in the already low fluorescence (Fig. 4C) and virtually no absorbance changes beyond 380 nm (Fig. 4D). The absorbance of AADP$^+$ at 330 nm is not observed when it is bound to the enzyme and thus appears only when AADP$^+$ is present in excess, i.e., in spectrum 6. The data for the change in fluorescence upon titration of $TrxR^{135S\text{-}PMA}_{138OH}$ and $TrxR^{135SH}_{138OH}$ with AADP$^+$ (Fig. 4A,C) conform to a rectangular hyperbola; the K_d values for the AADP$^+$ titrations are given in Table 1. Titration of AADP$^+$ into $TrxR^{135S}_{138S}$ or into $TrxR^{135S\text{-}PMA}_{138S\text{-}PMA}$ results in quenching of the fluorescence comparable to that observed with $TrxR^{135S\text{-}PMA}_{138OH}$; the values of the apparent dissociation constants are included in Table 1 for comparison.

Table 1. *Fluorescence quenching of untreated and treated enzymes by AADP$^+$*

Experiment	K_d for AADP$^+$ from fluorescence[a] (μM)	% Initial fluorescence at sat. AADP$^+$
$TrxR^{135S\text{-}MMTS}_{138OH}$	13.3 ± 1.5[b]	39
$TrxR^{135S\text{-}PMA}_{138OH}$	2.4 ± 0.2[c]	31
$TrxR^{135SH}_{138OH}$	6.8 ± 2.0	74
$TrxR^{135S}_{138S}$	8.0 ± 0.5	25
$TrxR^{135S\text{-}PMA}_{138S\text{-}PMA}$	5.4 ± 0.6	27

[a]Measured at 456 nm excitation and 540 nm emission.
[b]12.3 ± 0.6 μM by absorbance at 455 nm.
[c]3.6 ± 0.4 μM by absorbance at 455 nm.

Limited proteolysis

The FO and FR conformers would be expected to have some differences in surface-accessible residues and thus might have

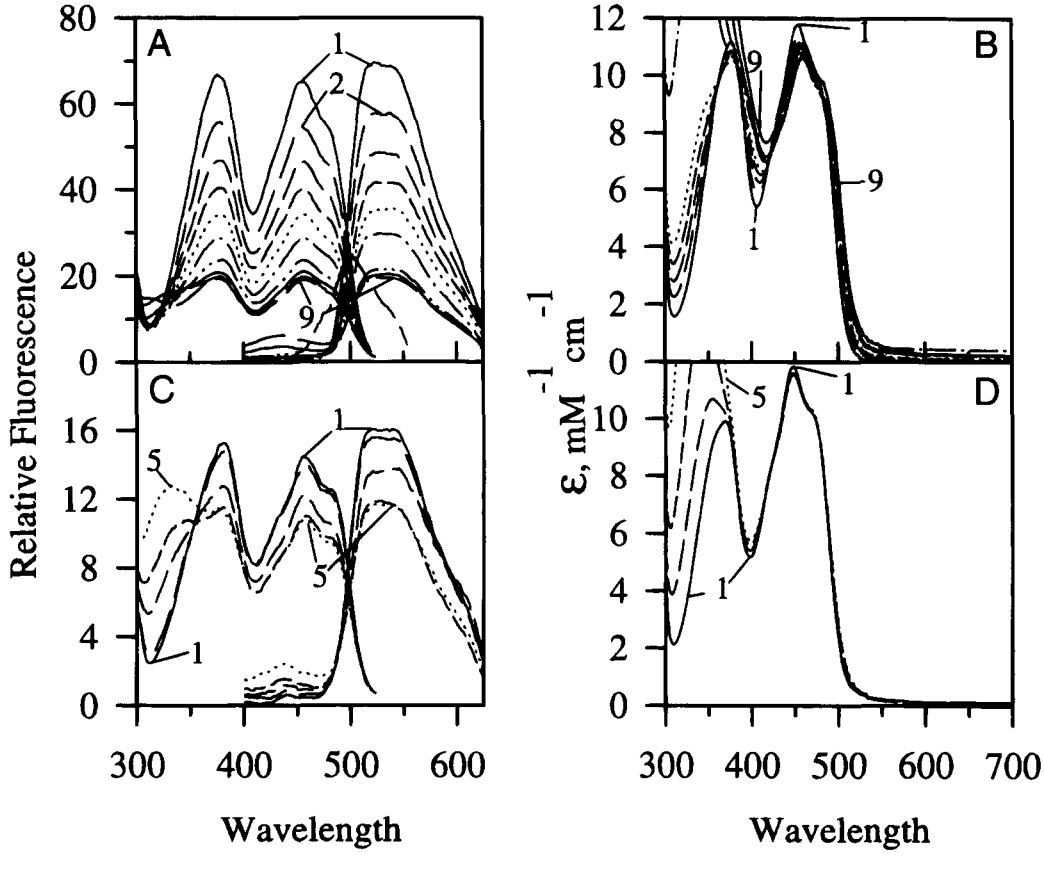

Fig. 4. Spectral analysis of AADP$^+$ titrations of $TrxR^{135SH}_{138OH}$ and $TrxR^{135S\text{-}PMA}_{138OH}$. **A,B:** Fluorescence and absorbance changes from titrating AADP$^+$ into 12.8 μM $TrxR^{135S\text{-}PMA}_{138OH}$. Nine spectra (1–9) were recorded at AADP$^+$ concentrations of 0, 3.3, 6.6, 9.9, 14.2, 21.7, 85.9, 191.4, and 392.2 μM. **C,D:** Fluorescence and absorbance changes resulting from addition of AADP$^+$ to 27.5 μM $TrxR^{135SH}_{138OH}$; five spectra (1–5) were recorded at AADP$^+$ concentrations of 0, 3.6, 27.5, 63.1, and 122 μM. In D, there was no change between spectrum 1 and 2. Note the difference in *y*-axis scales for A and C.

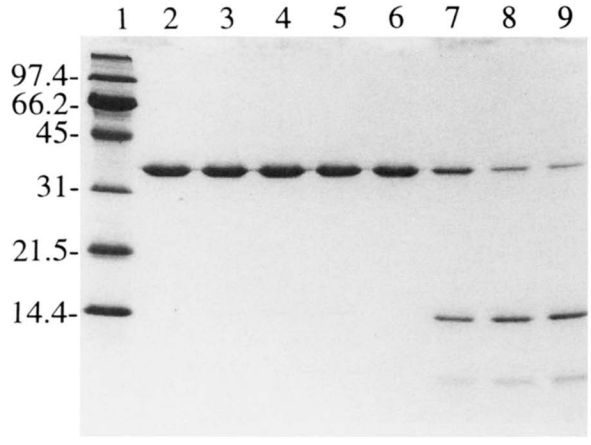

Fig. 5. Limited proteolysis of $TrxR|_{138OH}^{135SH}$ and $TrxR|_{138OH}^{135S\text{-}PMA}$. Lane 1, molecular weight standards; lanes 2–5, digests of $TrxR|_{138OH}^{135SH}$ for 0, 1.5, 3, and 6 h; lanes 6–9, digests of $TrxR|_{138OH}^{135S\text{-}PMA}$ for 0, 1.5, 3, and 6 h.

different proteolytic susceptibilities. This was tested by using EndoGluC protease in phosphate buffer, pH 7.6, which cuts on the carboxyl side of Glu and Asp amino acid residues. Exposure of $TrxR|_{138OH}^{135SH}$ or $TrxR|_{138OH}^{135S\text{-}PMA}$ to EndoGluC showed that $TrxR|_{138OH}^{135SH}$ was relatively unaffected by the protease, whereas the $TrxR|_{138OH}^{135S\text{-}PMA}$ was much more susceptible (Fig. 5). The same contrast was observed between $TrxR|_{138S}^{135S}$ and $TrxR|_{138S\text{-}PMA}^{135S\text{-}PMA}$. $TrxR|_{138S}^{135S}$ in the presence of 100 μM $AADP^+$ also did not digest (data not shown). We do not fully understand this result, which suggests that ligand binding does not restrict rotation in as absolute a way as does reaction with PMA.

It is of interest that the cleavage with EndoGluC causes only minor changes in the absorbance and fluorescence spectra of the enzyme, indicating that the 14.4-kDa fragment (see below) remains bound and FAD is bound normally, that is, in an apolar environment. The 14.4-kDa fragment constitutes a significant part of the FAD binding site (Waksman et al., 1994). Even the minor fluorescence changes during digestion are informative, however (data not shown). The fluorescence (emission and excitation) initially decreases to ca. 79%, and then increases to ca. 86% of that of unproteolyzed enzyme. This suggests that the flavin undergoes changes in polarity and that more than one peptide bond is broken.

The major SDS-PAGE band that occurs in the proteolysis of $TrxR|_{138OH}^{135S\text{-}PMA}$ runs at the same position as the 14.4-kDa standard. N-terminal sequence analysis of this fragment revealed NH_2-GTTKHS-, which is the amino terminus of the native enzyme. At the C-terminus of the fragment was a Glu, and the next amino acid from the end was a Glu with a small amount of Met/Ser. The only cleavage position capable of producing a fragment of the apparent size seen on SDS-PAGE with the observed end sequences is that at Ser 123-Glu 124-Glu 125. The major proteolytic product of EndoGluC digestion was generated by a single cut after Glu 125, which yielded a 13,569-kDa fragment, and a much smaller amount of cleavage occurred after Glu 124, to give a 13,440-kDa fragment.

Discussion

TrxR has classically been grouped with the pyridine nucleotide-disulfide oxidoreductases, including glutathione reductase and lipoamide dehydrogenase. The structures of these three enzymes are known. TrxR shares stretches of sequence homology, an FAD cofactor, a dimeric quaternary structure, some common aspects of mechanism of passage of reducing equivalents from NAD(P)H to a redox disulfide in the active site, as well as regions of structural similarity (Kuriyan et al., 1991; Williams, 1992, 1995; Waksman et al., 1994; Lennon & Williams, 1997). There are significant differences between TrxR and the other enzymes. TrxR is 150–240 amino acids shorter than the other enzymes, most of which is because TrxR lacks a separate interface domain that provides the contact surface between the monomers of the other enzymes.

Another major difference is in the arrangement of the active sites. Glutathione reductase and lipoamide dehydrogenase have the nicotinamide ring of bound NADPH and the nascent thiols of the active-site disulfide located on opposite sides of the isoalloxazine rings of FAD (*re* and *si* sides, respectively). The active-site dithiol is in a perpendicular geometry with respect to the flavin ring, with a charge transfer thiol close to the FAD and an interchange thiol further away (Karplus & Shultz, 1987; Mattevi et al., 1991). TrxR has the active-site disulfide adjacent to the FAD in a more parallel geometry, with Cys 138 only slightly closer to the *re* side of the flavin ring than Cys 135 (Fig. 1B). Thus, the nicotinamide ring of bound NADPH is displaced by the disulfide 17 Å from where it needs to be for efficient transfer of reducing equivalents to FAD (termed the FO conformation, Fig. 1A). There also is biochemical evidence that Cys 138 interacts directly with the FAD (Prongay et al., 1989; Prongay & Williams, 1990, 1992), and this was confirmed in the structure (Kuriyan et al., 1991; Waksman et al., 1994). It was postulated that the FAD and pyridine nucleotide domains could rotate 66° with respect to each other to allow the NADPH to move in close to the FAD and for the active-site disulfide/dithiol to move from the buried location to the surface (termed the FR conformation, Fig. 1A). In this putative model, the FAD and pyridine nucleotide domains of TrxR align structurally with the corresponding domains of glutathione reductase. This rotation was modeled as a rigid-body motion of one domain with respect to the other and there appeared to be no major hindrances to this domain movement (Waksman et al., 1994). Recently, the isolation of a stable complex between TrxR and Trx having properties consistent with its restriction to the putative FR conformation has been reported (Wang et al., 1996).

The proposed model of catalysis must include both the FR and FO conformations, the former where the flavin is being reduced by NADPH, and the latter where the flavin is oxidized by the active-site disulfide to yield a dithiol (Williams, 1995). Thus, although the FO form is found in the crystalline state, in solution it is assumed that there is an equilibrium between the two conformations. Indeed, evidence from rapid reaction kinetic studies of the reductive half reaction of $TrxR|_{138S}^{135S}$ and several mutants gave results consistent with $TrxR|_{138S}^{135S}$ being mostly in the FR conformation in solution, and $TrxR|_{138OH}^{135SH}$ mostly in the FO (Lennon & Williams, 1997; Williams, 1995; Lennon et al., 1997).

Three lines of evidence presented herein, when considered together, support the proposal that for $TrxR|_{138OH}^{135SH}$, the FR $\rightleftharpoons$ FO equilibrium is mostly in the FO conformation and for oxidized wild-type TrxR, the equilibrium favors the FR conformation. The first is the very low fluorescence of this mutant relative to wild-type TrxR. The crystal structure of both $TrxR|_{138S}^{135S}$ and $TrxR|_{138OH}^{135SH}$ were found to be identical except for a slight displacement of the Ser 138 oxygen; Cys 138 (or Ser 138) is slightly closer to the FAD C4a than Cys 135, a difference that appears somewhat exaggerated in Figure 1B (Waksman et al., 1994); therefore, the difference in

spectral properties between this mutant and wild-type TrxR is most likely due only to the presence of the serine in place of the cysteine. Of course, there also could be effects from the thiol of Cys 135: both disulfide active-site mutants of lipoamide dehydrogenase were found to have quenched fluorescence (Hopkins & Williams, 1995). Thus, the fluorescence quenching by the intrinsic quencher Ser 138 is interpreted as diagnostic for the FO conformer. In the proposed two-conformation model, the flavin environment of $TrxR|^{135SH}_{138OH}$ and $TrxR|^{135S}_{138S}$ should be nearly identical in the FR conformation, where Cys 135 and Ser 138 are far away from the FAD, and would be different in the FO conformation because Cys 135 and Ser 138 (or the disulfide of wild-type TrxR) are very close to the FAD (Fig. 1A).

The second line of evidence is the clear difference between the fluorescence excitation spectrum and the absorbance spectrum of $TrxR|^{135SH}_{138OH}$. The location of the absorbance and fluorescence excitation peaks as well as the low fluorescence intensity indicate that there are two populations of flavin environments. This point is addressed in more detail below.

The third line of evidence is the effect of the nonreducing NADP(H) analogue $AADP^+$ that causes profound changes in the spectral properties of $TrxR|^{135S\text{-}PMA}_{138OH}$ upon binding. In these studies, $AADP^+$ quenching of flavin fluorescence serves as a probe for the location of the pyridine nucleotide binding site relative to the FAD. If $AADP^+$ binds to TrxR in the FR conformation, the 3-aminopyridinium ring would be closely spaced and parallel to the *re* face of the isoalloxazine ring system of the FAD (Fig. 1A,B). This would result in significant quenching of the flavin fluorescence because complexes of heteroaromatic compounds with flavins are known to quench fluorescence (Weber, 1950; MacKenzie et al., 1969; Wu & McKormick, 1971; Ghisla et al., 1974). In contrast, binding of $AADP^+$ to TrxR in the FO conformation places it 17 Å away from the FAD, where it should have no effect on the flavin fluorescence. Thus, $AADP^+$ quenching is diagnostic for the FR conformer. In the case of $TrxR|^{135SH}_{138OH}$, addition of $AADP^+$ causes very little fluorescence quenching and no absorbance changes, results that are consistent with the FO form predominating for this mutant. This also indicates that $AADP^+$ does not perturb the FR/FO equilibrium significantly. This is an important point because the "trigger" for rotation, if there is one, is still unknown.

Evidence that $AADP^+$ binds to $TrxR|^{135SH}_{138OH}$ in a manner that is unproductive for quenching is seen by comparison of the 330-nm region of Figure 4A and C. $AADP^+$ is fluorescent with an excitation maximum at 330 nm and emission maximum at 420 nm, with a small amount of emission at 540 nm (Tu, 1981). At each $AADP^+$ addition to $TrxR|^{135SH}_{138OH}$ (Fig. 4C), fluorescence excitation at 330 nm increases (540 nm emission), whereas for $TrxR|^{135S\text{-}PMA}_{138OH}$ (Fig. 4A), the fluorescence excitation in this region decreases until the last few additions, where saturation is reached. Thus, $AADP^+$ added to $TrxR|^{135S\text{-}PMA}_{138OH}$ is bound such that FAD fluorescence and $AADP^+$ fluorescence are mutually quenched. $AADP^+$ added to $TrxR|^{135SH}_{138OH}$ is bound such that FAD fluorescence and $AADP^+$ fluorescence are not quenched or is not bound at all.

The fluorescence results (Figs. 2, 4), which indicate intrinsic quenching from Ser 138 in the FO conformation and extrinsic quenching by $AADP^+$ in the FR form, support the conclusion that reaction of $TrxR|^{135SH}_{138OH}$ with MMTS or PMA forces the FO $\rightleftharpoons$ FR equilibrium toward the FR side. When $TrxR|^{135SH}_{138OH}$ is reacted with MMTS or PMA, a thiomethyl or a phenylmercuric adduct is made on the remaining thiol of Cys 135. Although this is the only thiol of $TrxR|^{135SH}_{138OH}$ that is accessible to solvent in the FO state (using a surface calculation with a 1.4 Å radius; Waksman et al., 1994), it can also react with these reagents when it is more exposed in the FR conformation. We have modeled a thiomethyl adduct to Cys 135 in the FO crystal structure: such an adduct is forbidden in the tightly packed space without significant side-chain or main-chain adjustment. However, this steric argument is probably not the only factor favoring the FR conformation. Changes in the polarity of the active site could be important. In glutathione reductase, the thiol nearer the flavin reacts with PMA when the thiol further from the flavin is alkylated, and the reaction may have been favored by the apolar site (Arscott et al., 1981). The juxtaposition of the pyridine nucleotide binding and flavin binding domains in glutathione reductase is the same as in the FR conformation of thioredoxin reductase (Waksman et al., 1994). It is therefore reasonable to assume that, for $TrxR|^{135S\text{-}MMTS}_{138OH}$ or $TrxR|^{135S\text{-}PMA}_{138OH}$, the FO conformation becomes unfavorable energetically and the FR form would predominate. As the reaction proceeds, Ser 138 moves away from the FAD with a concomitant fluorescence increase, and the pyridine nucleotide binding site moves adjacent to the flavin ring.

It follows from the discussion above that the FO $\rightleftharpoons$ FR equilibrium would be shifted to the FR side for a small thiomethyl adduct and would be almost completely in the FR conformation with a large adduct, such as a phenylmercuric moiety. $TrxR|^{135S\text{-}PMA}_{138OH}$ had higher fluorescence than $TrxR|^{135S\text{-}MMTS}_{138OH}$ (ca. 17%). The data in Table 1 show that $TrxR|^{135S\text{-}PMA}_{138OH}$ had a smaller K_d for $AADP^+$ than that for $TrxR|^{135S\text{-}MMTS}_{138OH}$. Both these results indicate that more of the PMA-treated enzyme was in a conformation that was productive for fluorescence quenching by $AADP^+$, that is, the FR form. These same arguments apply to the case of wild-type enzyme, where presumably two adducts are formed on the free thiols of the reduced enzyme (O'Donnell & Williams, 1985).

Analysis of the absorbance and fluorescence spectra have provided evidence that $TrxR|^{135SH}_{138OH}$ is a mixture of two conformers. Comparison of the absorbance (Fig. 3, solid line) and fluorescence excitation spectra (Fig. 2, spectrum 1) show that the fluorescence has peaks at 380 nm and 456 nm, whereas the absorbance peaks were found at 368 nm and 448 nm. Addition of PMA to $TrxR|^{135SH}_{138OH}$ resulted in pronounced absorbance changes, eventually giving a spectrum that resembled the fluorescence excitation spectrum. The fluorescence excitation spectrum did not change upon addition of PMA, only the intensity increased. Because the fluorescence excitation spectrum of a fluorophore will resemble the absorbance spectrum, these findings support the hypothesis that $TrxR|^{135SH}_{138OH}$ is a mixture of two conformational species, one that is nonfluorescent with absorbance peaks at 368 nm and 448 nm and greatly predominates (FO), and a second species that has high fluorescence and absorbance peaks at 378 nm and 455 nm (FR). Addition of PMA, because of steric restrictions of the phenylmercuric adduct in the FO conformation, forces the equilibrium toward the FR form, which leads to the observed spectral perturbations.

The absorbance spectral changes that resulted upon addition of PMA to $TrxR|^{135SH}_{138OH}$, which presumably removes the neighboring Ser 138 or Cys 135 by a conformational change (Fig. 3), lead to the tentative conclusion that the fluorescence quenching is mostly due to static (ground state) quenching rather than dynamic (collisional) quenching. This conclusion is reinforced by the results with $AADP^+$ binding to $TrxR|^{135S\text{-}PMA}_{138OH}$ (Fig. 4B) or to $TrxR|^{135S\text{-}PMA}_{138S\text{-}PMA}$ or $TrxR|^{135S}_{138S}$ (data not shown). Thus, the quenching by $AADP^+$ that was observed was mainly due to the formation of a ground state complex with low or no fluorescence rather than quenching by increased depopulation of FAD molecules in the excited state. Formation of low

or nonfluorescent ground-state complexes is expected to be accompanied by changes in the absorbance spectrum of the fluorophore (Lakowicz, 1983), and this is observed in Figure 4A (excitation) and B. In the case of AADP$^+$ quenching of $TrxR|_{138OH}^{135S\text{-}PMA}$, $TrxR|_{138S\text{-}PMA}^{135S\text{-}PMA}$, or $TrxR|_{138S}^{135S}$, a complex was formed that was only 25–31% as fluorescent as the enzyme without AADP$^+$ (Table 1).

The AADP$^+$ quenching results are in agreement with the proposal that $TrxR|_{138S}^{135S}$ exists in solution mostly in the FR conformation (Lennon & Williams, 1997; Lennon et al., 1997). Titration of $TrxR|_{138S}^{135S}$ with AADP$^+$ caused a large reduction in fluorescence (Table 1). Nearly identical results were observed with $TrxR|_{138S\text{-}PMA}^{135S\text{-}PMA}$.

Limited proteolysis by EndoGluC showed that PMA treatment of both $TrxR|_{138OH}^{135SH}$ (Fig. 5) and reduced $TrxR|_{138S}^{135S}$ rendered them much more susceptible to the protease. Sequencing of the ends of the 14-kDa proteolytic fragment revealed that it came from the amino-terminal end of the enzyme from a single cut after Glu 124 or Glu 125. Examination of the FO and putative FR structures showed that the Glu 124–Glu 125 region is on the surface: thus, the difference in proteolysis is not due to this region being exposed in the FR form and buried in the FO conformation. This leaves three plausible explanations: (1) the rapid FO $\rightleftharpoons$ FR equilibrium (65 s^{-1} in each direction; Lennon, 1995) in the untreated enzymes does not allow enough time for the relatively slow-acting protease to work; PMA treatment constricts the enzymes to the FR form so the EndoGluC has time to act; (2) the PMA adduct causes strain on the protein backbone, which destabilizes some regions, rendering them susceptible to the protease; and (3) the protease cleaves at another Glu or Asp residue that is exposed only in the FR conformation and this leads to subsequent rapid fragmentation. We favor the first suggestion, that proteolytic susceptibility is not an indicator of the FR conformation, but rather is characteristic of conformational restriction in the PMA-treated enzymes. The minor changes in the flavin fluorescence during proteolysis indicate that the polarity of the isoalloxazine ring is not altered significantly by the digestion.

We have presented evidence for two conformational states of TrxR based on the distinct flavin environments found in each: proximity of Ser 138 in the FO form and proximity of the pyridine nucleotide binding site in the FR form. The following conclusions can be summarized from these results in terms of the conformational equilibrium model diagrammed in Figure 1. For $TrxR|_{138OH}^{135SH}$, the equilibrium is mostly in the FO conformation, as demonstrated by the intrinsic quenching of flavin fluorescence by Ser 138 and the inability of AADP$^+$ to affect the spectral properties significantly. Reaction of the remaining active-site Cys 135 with a thiol-specific reagent, because of steric limitations in the FO conformation, forces the equilibrium to the FR form and relieves the flavin fluorescence quenching. The flavin is now adjacent to the pyridine nucleotide binding site, where its fluorescence can be quenched and spectral properties perturbed by AADP$^+$ binding. Limited proteolysis results are consistent with the existence of an equilibrium between the two forms, and, when TrxR is restricted conformationally to the FR form, the resulting structure is now sensitive to digestion.

Materials and methods

Reagents and enzymes

DTNB, AADP$^+$, Endoproteinase Glu-C, and NADPH were purchased from Sigma. DTT was from ICN. All other reagents were of the finest quality available.

Overexpression and purification of $TrxR|_{138S}^{135S}$ and $TrxR|_{138OH}^{135SH}$

$TrxR|_{138S}^{135S}$ was expressed from recombinant plasmid pTrR301 as described previously (Mulrooney, 1997). The $TrxR|_{138OH}^{135SH}$ mutant was expressed from a new plasmid construct as follows. Plasmid pTrR301, which has the cloned and highly expressed wild-type TrxR, was digested with restriction enzyme *Bsg* I and the resulting largest fragment was purified from an agarose gel as described previously (Mulrooney, 1997). Likewise, plasmid pAJP2 (Prongay et al., 1989), which contained the Cys 138 to Ser mutation, was also digested with *Bsg* I and the resulting small fragment was recovered from an agarose gel. The two fragments were then ligated and the resulting new plasmid, designated pTrR336, was used for production of $TrxR|_{138OH}^{135SH}$. For growth of the recombinant enzyme for purification, plasmid pTrR336 was transformed into *E. coli* strain A326, which has an insertion in the genomic TrxR gene (Russel & Model, 1986), but often failed to yield colonies. It was found that transforming strain A326 first with a compatible plasmid, which contains a cloned *lac*I^q gene that overproduces the lac repressor (pUH7; Henrich & Plapp, 1986) and presumably moderates expression from pTrR336, allowed for successful transformation and mutant enzyme expression from pTrR336. Cultures of 300 mL of were grown and the enzyme was purified as described previously (Mulrooney, 1997) with typical yields ranging from 70 to 126 mg.

Fluorescence measurements

Fluorescence spectra were recorded on a Perkin-Elmer MPF-44B fluorimeter at 25 °C. Spectra were corrected for variations in lamp intensity and photomultiplier sensitivity. The fluorimeter was adjusted relative to a standardized FAD solution before each scan to correct for instrument drift.

PMA and MMTS reactions of $TrxR|_{138S}^{135S}$ and $TrxR|_{138OH}^{135SH}$

$TrxR|_{138OH}^{135SH}$ was pre-treated with 20 equivalents of DTT for 20 min at 25 °C to remove potential sulfenic acid adducts on Cys 135. The enzyme was then washed extensively in a Centricon-30 (Amicon) with 50 mM phosphate, 1.5 mM EDTA, pH 7.6 (buffer B). This DTT-treated $TrxR|_{138OH}^{135SH}$ was used the same day for MMTS or PMA reactions. Reaction of PMA with $TrxR|_{138S}^{135S}$ was performed by first reducing the enzyme with five equivalents of NADPH under anaerobic conditions and then adding three equivalents of PMA. The reaction was allowed to proceed 10 min before opening to air and then washed extensively in a Centricon-30 using buffer B.

Stopped-flow rapid reaction kinetics

Kinetics of PMA reaction with $TrxR|_{138OH}^{135SH}$ were performed in buffer B at 25 °C in a stopped-flow apparatus that was described previously (Lennon & Williams, 1995), but had been modified with an observation cell to measure fluorescent light at a right angle to the incident light. Input light from a xenon lamp was filtered through a 450-nm bandpass filter and right angle output light was filtered through a 480-nm cutoff filter. The output light was quantitated by a photomultiplier and recorded. The enzyme was pre-treated with DTT as described above and the concentration in the stopped flow was 10.4 μM after mixing. The PMA was dissolved in buffer B and had concentrations of 20, 40, 80, and 160 μM after mixing.

AADP$^+$ titrations

PMA- or MMTS-treated enzymes were prepared by reacting $TrxR|^{135S}_{138S}$ or $TrxR|^{135SH}_{138OH}$ as described above. Samples of enzyme or treated enzyme were then placed into a 1-mL fluorescence cuvette and both absorbance and fluorescence spectra were recorded after each addition of AADP$^+$. Binding constants were calculated from fitting the fluorescence excitation peak (456 nm) measurement at each AADP$^+$ concentration to a rectangular hyperbola (SigmaPlot for Windows 2.0, Jandel Scientific, San Rafael, California).

Limited proteolysis

Untreated or PMA-treated enzymes were reacted with EndoGluC at a ratio of 20:1 (w/w) at 37 °C in buffer B. For analytical purposes, aliquots were removed at the desired time points and the reaction was stopped by addition of 0.3 volume of 8% trichloroacetic acid and frozen until needed for SDS-PAGE analysis. For preparative gels to obtain the small fragment for sequencing, reactions were treated to kill the protease activity by adding SDS to a final concentration of 2% (w/v) and 2-mercaptoethanol to a final concentration of 10% (v/v) and boiling for 4 min (Cleveland et al., 1977). The samples were run on SDS-PAGE using an 18% acrylamide (29.2:0.8; acrylamide:bisacrylamide) running gel. N-terminal and C-terminal amino acid sequence analysis of the 14-kDa proteolytic fragment was performed at the University of Michigan Biomedical Research Core Facility using automated procedures.

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