Enzyme-Monitored Turnover of *Escherichia coli* Thioredoxin Reductase: Insights for Catalysis†

Brett W. Lennon and Charles H. Williams, Jr.*

Department of Biological Chemistry, University of Michigan, and Department of Veterans Affairs Medical Center, Ann Arbor, Michigan 48105

Received October 23, 1995; Revised Manuscript Received January 25, 1996⊗

ABSTRACT: Thioredoxin reductase from *Escherichia coli* is a member of the pyridine nucleotide-disulfide oxidoreductase family, and contains one FAD and one redox-active disulfide per subunit. It is known that two other well-studied members of this family, lipoamide dehydrogenase and glutathione reductase, cycle between the two electron-reduced and fully oxidized forms in catalysis. Enzyme-monitored turnover shows that the spectrum of thioredoxin reductase during turnover represents fully reduced flavin with NADP(H) bound. Whether the pyridine nucleotide bound is NADPH or $NADP^+$ is dependent on the concentration of each species, i.e., how far turnover has progressed. It is also shown that the midpoint potentials of this enzyme are increased through the differential binding of $NADP^+$ to the oxidized and reduced forms of the enzyme. When combined with other kinetic and oxidation/reduction studies of this enzyme, these results indicate that thioredoxin reductase cycles between the four-electron-reduced and two-electron-reduced forms in catalysis, and that it does so with pyridine nucleotide bound. These results clarify the mechanism of thioredoxin reductase in relation to the known structure of the enzyme, and provide support for earlier work in which we proposed that this enzyme utilizes a ternary complex mechanism in catalysis.

Thioredoxin reductase (EC 1.6.4.5) is a member of the pyridine nucleotide-disulfide oxidoreductase family (Williams, 1992). Thioredoxin reductase from *Escherichia coli* is a homodimer; each monomer of $M_r = 35\,300$ contains one FAD and one redox-active disulfide (Moore et al., 1964; Russel & Model, 1988; Zanetti & Williams, 1967). The reaction catalyzed by this enzyme is the reduction of a small protein ($M_r = 11\,700$), thioredoxin, by NADPH (Moore et al., 1964; Holmgren, 1968). Electrons are passed from NADPH to the FAD, from reduced flavin to the redox-active disulfide of thioredoxin reductase, and then from the newly formed enzyme dithiol to the disulfide of thioredoxin (Holmgren, 1985).

The X-ray crystal structure of thioredoxin reductase has been solved (Waksman et al., 1994). This structure shows the redox-active disulfide as being adjacent to the *re* face of the isoalloxazine ring of FAD, a position which is favorable for hydride transfer between the flavin and the disulfide (Figure 1, lower left panel). Since this conformation allows flavin oxidation by the enzyme disulfide, it is referred to as the FO conformation. However, the nicotinamide ring of NADPH is not able to approach the FAD, and the buried position of the enzyme active site sulfurs appears to prevent their interaction with the bound substrate, thioredoxin. If the NADPH domain of thioredoxin reductase is rotated 66° relative to the FAD domain using the graphics terminal, the two domains become positioned as they are in glutathione reductase, a model member of this enzyme family (Waksman et al., 1994) (Figure 1, upper panel). This unimpeded rotation brings the nicotinamide ring of NADPH adjacent to the *re* face of the flavin ring, which is favorable for hydride transfer from NADPH to the flavin. Since this conformation allows flavin reduction by pyridine nucleotide, it is referred to as the FR conformation (Figure 1, lower right panel). In addition, the active site dithiol is presented to a potential thioredoxin binding site by the same rotation. Thus, the occurrence and reversal of the proposed conformational change enable NADPH and the active site sulfurs to alternately interact with the flavin. The current hypothesis is that the enzyme exists in solution in an equilibrium mixture of both the FO and FR conformations (Lennon, 1995). It is not known what effect the binding of NADPH or enzyme reduction may have on the proposed conformational change. Evidence exists which does suggest that the binding of thioredoxin favors the FR conformation (Wang et al., 1996), presumably by binding the FR conformation and pulling the equilibrium to favor that conformation.

We have referred in two previous publications to the conformation observed in the crystal structure as the unrotated conformation and to the putative conformation as the rotated conformation (Lennon & Williams, 1995; Williams, 1995). We regret this choice which will have relevance only, we hope, until the putative conformation is observed. We propose, therefore, to refer to the observed and putative conformations as FO and FR, respectively, as described in the previous paragraph.

It is known that in two of the other members of this enzyme family, lipoamide dehydrogenase and glutathione reductase, there is a distinct anaerobically stable two-electron-reduced species containing oxidized flavin and a reduced

† This research was supported by the Department of Veterans Affairs (C.H.W.), by NIGMS Grant GM21444 (C.H.W.), and by the Pharmacological Sciences Training Program, University of Michigan (B.W.L.), which is funded by the National Institutes of Health.

* To whom correspondence should be addressed at Medical Research Service, 151, VA Medical Center, 2215 Fuller Rd., Ann Arbor, MI 48105. Telephone: (313) 769-7100, ext 5611. FAX: (313) 761-7693.

⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1996.

0006-2960/96/0435-4704$12.00/0

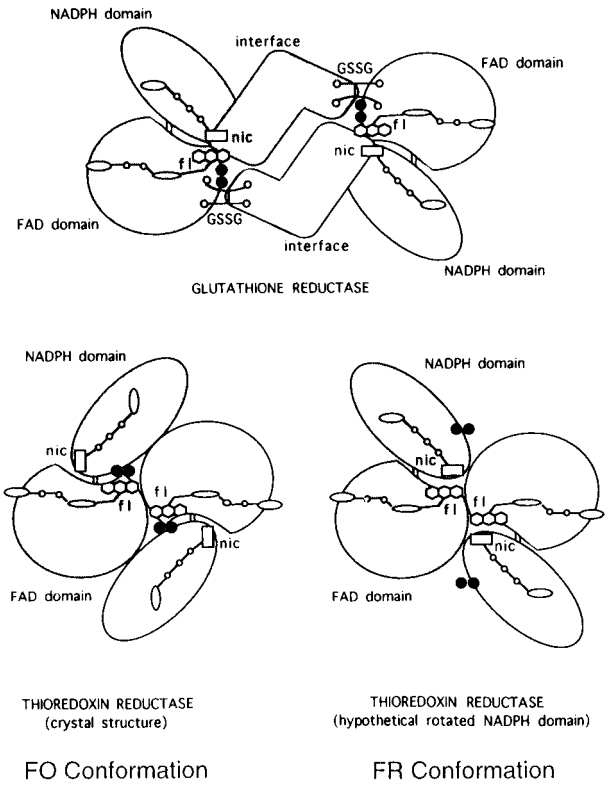


FIGURE 1: Comparison of the observed and proposed conformations of thioredoxin reductase. From Waksman et al. (1994), with permission. In the upper panel is a representation of glutathione reductase, a model member of this enzyme family. In the lower two panels are representations of thioredoxin reductase. Note that the orientations of these drawings are such that the FAD domains of thioredoxin reductase can be overlaid with those of glutathione reductase. In the FO conformation of thioredoxin reductase observed in the crystal structure (lower left panel), the enzyme active site disulfide (black circles) is juxtaposed to accept electrons from the isoalloxazine ring of the flavin (fl). The nicotinamide ring of NADP(H) (nic) is not able to approach the flavin, and the active site disulfide is buried so that it does not seem to be accessible by thioredoxin. In the proposed FR conformation (lower right), the NADPH domain has been rotated so that it occupies a position relative to the FAD domain which is similar to that observed in glutathione reductase (cf. the structure of glutathione reductase in the upper panel). In this conformation, the nicotinamide ring is able to approach the isoalloxazine ring of the FAD, and the active site disulfide is more accessible by thioredoxin.

disulfide (Williams, 1992). This is a direct consequence of the substantial separation between the midpoint potentials of the E_{ox}/EH_2 and EH_2/EH_4 couples. In those enzymes, catalysis proceeds from this two-electron-reduced enzyme to oxidized enzyme, and then back to the two-electron-reduced state. For thioredoxin reductase, it can be calculated based on the pH dependence of the published redox data that the midpoint potentials of the flavin and disulfide are separated by only 7 mV at pH 7.6 (O'Donnell & Williams, 1983). Thus, partially reduced enzyme is a mixture of four species: oxidized enzyme, enzyme containing an active site disulfide and reduced flavin, enzyme containing an active site dithiol and oxidized flavin, and four-electron-reduced enzyme containing an active site dithiol and reduced flavin. It is not clear which species are active in catalysis.

To address this question, enzyme-monitored turnover was used. Enzyme-monitored turnover with flavoenzymes involves the reaction of an enzyme of interest with its substrates where the progress of the reaction is followed by observing the enzyme flavin absorbance. This has been used to obtain steady-state kinetic parameters (Gibson et al., 1964; Lockridge et al., 1972), but this particular use of the technique was not employed for thioredoxin reductase. Instead, enzyme-monitored turnover was used simply to observe the flavin spectral characteristics during turnover. Previous work in this laboratory has shown that the presence of pyridine nucleotides decreases the rate of the reoxidation of reduced thioredoxin reductase by oxidized thioredoxin to essentially k_{cat} (Lennon & Williams, 1995). This was interpreted as evidence in favor of a ternary complex in catalysis by thioredoxin reductase. The enzyme-monitored turnover experiment in the current work not only clarifies the redox state of thioredoxin reductase during turnover, but the capability to collect full spectra on the millisecond time scale enabled us to gather evidence which confirms the proposal that a ternary complex is the key intermediate in catalysis by thioredoxin reductase (Lennon & Williams, 1995). This clarification of the mechanism is consistent with the proposal that a large conformational change must be a step in catalysis based on the known structure of the enzyme (Waksman et al., 1994).

MATERIALS AND METHODS

Reagents. $NADP^+$ (Sigma grade) and NADPH (type III, enzymatically reduced) were purchased from Sigma. Sodium dithionite was purchased from Fluka Chemical Corp. All other reagents and buffers were of the highest quality available.

E. coli Thioredoxin Reductase Purification. Wild-type thioredoxin reductase was prepared as previously described (Lennon & Williams, 1995), except that the enzyme was eluted from the Pharmacia Q Sepharose column with a linear gradient of 0−0.8 M NaCl over 7 column volumes. The enzyme eluted at about 0.45 M salt.

One preparation of enzyme was grown in strain XL1-B[pTRR201] as previously described (Mulrooney & Williams, 1994). This enzyme contained an extraneous mutation, that of Glu^{70} to Asp. Since this is a conservative mutation, and Glu^{70} lies on the surface of the enzyme away from the active site, this mutation should have no effect on the results presented here. The wild-type (E70D) enzyme was only used in the turnover experiment (see below). It should be noted that the results of several experiments studying the rapid reaction of true wild-type enzyme and the wild-type (E70D) enzyme with NADPH produced the same results as observed at 456 nm (Williams et al., 1991; Lennon, 1995; and data not shown). This gives support to the conclusion that the extraneous mutation does not affect the enzyme catalytic function.

Thioredoxin Purification. Thioredoxin was purified from *E. coli* as previously described (Lennon & Williams, 1995).

Stopped-Flow Spectrophotometry. Anaerobiosis of enzyme and substrate samples was achieved, and rapid reaction studies were carried out using the techniques and equipment previously described (Lennon & Williams, 1995).

Enzyme-Monitored Turnover. The experiment was done in 0.1 M sodium/potassium phosphate, pH 7.6 at 1 °C. Anaerobic oxidized enzyme (11 μM), to which 1.5 mM thioredoxin had been added, was placed in one syringe of the stopped-flow instrument. The second syringe contained anaerobic 1.5 mM oxidized thioredoxin and either 165 μM or 552 μM NADPH. Thioredoxin was present in the enzyme

Table 1: Midpoint Potentials for the Redox Couples Involved in Turnover with Thioredoxin Reductase[a]

redox couple	E_m (mV)
enzyme-FAD/$FADH_2$, active site sulfurs as a disulfide[b]	−263
enzyme-FAD/$FADH_2$, active site sulfurs as a dithiol[b]	−280
enzyme-$(S)_2/(SH)_2$, flavin oxidized[b]	−270
enzyme-$(S)_2/(SH)_2$, flavin reduced[b]	−287
$NADP^+$/NADPH[c]	−313
thioredoxin-$(S)_2/(SH)_2$[d]	−282

[a] Values shown are for pH 7.6 and 1 °C. Corrections for pH and temperature, if necessary, were applied based on data in the references cited (see Materials and Methods). [b] Calculated from O'Donnell and Williams (1983). [c] Calculated from Engel and Dalziel (1967), corrected for the ionic strength effect of 0.1 M sodium/potassium phosphate buffer. [d] Calculated from data in Moore et al. (1964), Krause et al. (1991), and Engel and Dalziel (1967).

sample to prevent any mixing artifacts that might arise from the use of such a high concentration of protein substrate. The enzyme was rapidly mixed with the two substrates in the second syringe, the absorbance of the flavin during turnover was followed at 456 nm, and diode array spectra were taken. The reaction was repeated 3−4 times at each concentration of NADPH. Final concentrations were 5.5 μM enzyme, 1.5 mM thioredoxin, and 82.4 μM or 276 μM NADPH.

Titration of Reduced Thioredoxin Reductase with Oxidized Thioredoxin and NADP$^+$. 1.5 mL of 25 μM enzyme in 0.1 M sodium/potassium phosphate, pH 7.6, was reduced anaerobically by 2.1 equiv of sodium dithionite as described previously (Lennon & Williams, 1995). The reduced enzyme was then titrated at 25 °C with up to 15 equiv of anaerobic oxidized thioredoxin by addition from a Hamilton gas-tight syringe, and the spectral changes caused by the reoxidation of flavin by thioredoxin were observed in a Milton Roy Spectronic 3000 diode array spectrophotometer. Up to 23 equiv of $NADP^+$ was then added from a Hamilton gas-tight syringe, and the flavin spectral changes were observed.

Calculation of Midpoint Potentials. In order to compare the midpoint potentials of the various couples involved in turnover by thioredoxin reductase, it was necessary to calculate the values of the midpoint potentials under the conditions of the turnover experiments presented in this work (pH 7.6 and 1 °C). The values are summarized in Table 1. The midpoint potentials of thioredoxin reductase at pH 7 and 12 °C are as follows: FAD/$FADH_2$ (active site sulfurs as a disulfide), −243 mV; FAD/$FADH_2$ (active site sulfurs as a dithiol), −260 mV; $(S)_2/(SH)_2$ (flavin oxidized), −254 mV; $(S)_2/(SH)_2$ (flavin reduced), −271 mV (O'Donnell & Williams, 1983). The values of these couples were corrected to pH 7.6 by applying published pH dependencies of −59.2 mV/pH for the flavin couples, and −52 mV/pH for the disulfide/dithiol couples (O'Donnell & Williams, 1983). No data exist regarding the temperature dependence of either the flavin or the disulfide/dithiol couples of thioredoxin reductase, and we were not able to locate such values for any flavoenzyme or disulfide/dithiol couples in the literature. We have used a correction factor of −1.39 mV/°C for the standard hydrogen electrode (Clark, 1960)[1] to correct these values to 1 °C.

[1] Some temperature correction factors of interest are: standard hydrogen electrode, −1.39 mV/°C (Clark, 1960); NADH, −1.3 mV/°C (Clark, 1960); dithionite, −1.6 mV/°C (Mayhew, 1978); methyl viologen, −0.6 mV/°C (Watt & Burns, 1975).

The E_m of the $NADP^+$/NADPH couple is sensitive to ionic strength (Engel & Dalziel, 1967). In that reference, the E_m at pH 7 and 25 °C, extrapolated to $I = 0$, is calculated to be −335 mV. The experiments reported in the current work were done in 0.1 M sodium/potassium phosphate buffer at pH 7.6. This buffer contains 86.6 mM K_2HPO_4 and 13.4 mM NaH_2PO_4, which corresponds to $I = 0.282$. Using the data from Engel and Dalziel (1967), the E_m for the $NADP^+$/NADPH couple at pH 7, 25 °C, and $I = 0.282$ is calculated to be −327 mV. A pH dependence for the $NADP^+$/NADPH couple of −29.6 mV/pH at 25 °C was assumed (two electrons, one proton) (Clark, 1960), and a −1.3 mV/°C correction factor for NADH was applied (Clark, 1960) to obtain a calculated value of $E_m = -313$ mV at pH 7.6 and 1 °C.

The E_m for the thioredoxin-$(S)_2/(SH)_2$ couple has been determined (Moore et al., 1964; Krause et al., 1991). The data from Moore et al. (1964) were obtained at 21 °C in 98 mM Tris base brought to pH 7, 8, or 9 with HCl, and which also contained 9.8 mM EDTA. Utilizing published pK_a values for Tris (Stoll & Blanchard, 1990) and EDTA (O'Sullivan, 1969), the ionic strength for this system was estimated to be 0.098 at pH 7.0, 0.067 at pH 8.0, and 0.021 at pH 9.0. The data of Engel and Dalziel (1967) were then used to calculate the midpoint potential of the $NADP^+$/NADPH couple at these ionic strengths, and the values were adjusted to the respective pH values and to 21 °C as described above (−323 mV at pH 7.0, −353 mV at pH 8.0, and −386 mV at pH 9.0). These values were used in conjunction with the equilibrium data from Moore et al. (1964) to calculate the E_m value of thioredoxin at those pH values. The resulting plot of E_m against pH for thioredoxin (slope = −58 mV/pH) was used to obtain the E_m at pH 7.6. As was the case with thioredoxin reductase, since no temperature correction factor exists for thioredoxin, this value was corrected from 21 °C to 1 °C using the −1.39 mV/°C correction factor for the standard hydrogen electrode (Clark, 1960) to obtain an E_m value of −281 mV for thioredoxin at pH 7.6 and 1 °C.

A similar approach was used with the data from Krause et al. (1991). The E_m value of the $NADP^+$/NADPH couple under the conditions in that work is calculated to be −327 mV at pH 7.0 and 25 °C using the data of Engel and Dalziel (1967). Krause et al. (1991) reported a $\Delta E_m = 45$ mV between thioredoxin and $NADP^+$/NADPH, and so the E_m for thioredoxin under the same conditions is calculated to be −282 mV. Using the pH correction (−58 mV/pH) derived above from the data of Moore et al. (1964) and once again correcting to 1 °C using the standard hydrogen electrode value of −1.39 mV/°C (Clark, 1960), the E_m for thioredoxin at 1 °C and pH 7.6 is calculated to be −283 mV, which is in excellent agreement with the result based on the data from Moore et al. (1964). The average of this value and that obtained from Moore et al. (1964) (see above) is −282 mV. The fact that these values obtained from data collected at different temperatures are so similar suggests that the temperature correction factor of −1.39 mV/°C for the standard hydrogen electrode does not have significant error when used for thioredoxin.

RESULTS

Enzyme-Monitored Turnover. The changes in extinction at the flavin peak (456 nm) observed in the enzyme-

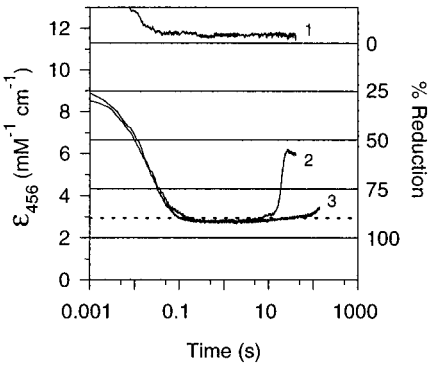


FIGURE 2: Reduction state of the flavin in thioredoxin reductase during turnover. Final conditions were 5.5 μM enzyme, 1.5 mM thioredoxin, and various concentrations of NADPH in 0.1 M sodium/potassium phosphate, pH 7.6, 1 °C. Thioredoxin was included in both enzyme and substrate syringes to minimize mixing artifacts. The reduction state of the flavin was monitored at 456 nm. Curve 1, oxidized enzyme mixed rapidly with buffer to illustrate the level of starting extinction. The decrease in extinction in the early part of this curve is an artifact caused by the mixing of the high concentration of thioredoxin present in the enzyme sample with buffer. The starting extinction level is slightly higher than the 11 300 M^{-1} cm^{-1} of free oxidized enzyme due to the perturbation of the flavin spectrum upon binding thioredoxin. Curve 2, 82.4 μM NADPH. Curve 3, 276 μM NADPH. The dashed line represents 90% reduction.

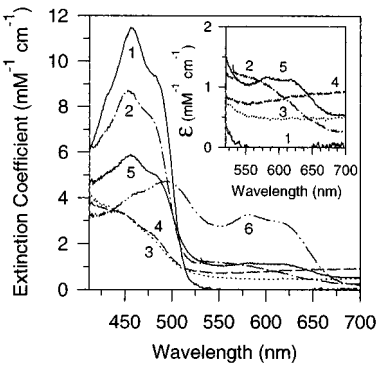


FIGURE 3: Spectra taken during turnover of thioredoxin reductase. Conditions were 5.5 μM enzyme, 1.5 mM thioredoxin, and 82.4 μM NADPH in 0.1 M sodium/potassium phosphate, pH 7.6, 1 °C. Spectra are (1) oxidized enzyme, (2) dead time spectrum (2.9 ms), (3) spectrum during turnover (192 ms), (4) spectrum at the end of turnover (10 s), (5) 2 min after start of reaction, and (6) 5 min after start of reaction. The sample was exposed to white light over the 3 min following the previous spectrum. Inset: Magnification of the long-wavelength region of the first five spectra of the main figure (oxidized enzyme through the 2 min spectrum).

monitored turnover experiment are shown in Figure 2. When oxidized thioredoxin reductase is rapidly mixed with a mixture of limiting NADPH and excess thioredoxin at 1 °C, the flavin is quickly reduced by the NADPH, as evidenced by the decreasing extinction. Reduction is followed by turnover, during which the flavin is 92% reduced (the flat portion of the traces). With 82.4 μM NADPH, turnover lasted from about 0.1 s to 10 s (curve 2, Figure 2). Increasing the concentration of NADPH to 276 μM clearly extends the duration of turnover (curve 3, Figure 2), but does not affect the level of reduction of the flavin during turnover. After the NADPH was consumed, the excess thioredoxin was able to oxidize the flavin, as shown by the increase in flavin peak extinction. Note that even with a large excess of oxidized thioredoxin present at the end of turnover, the enzyme is not fully reoxidized. This is in contrast to what is observed when reduced enzyme is oxidized by thioredoxin in the absence of pyridine nucleotide, where ~95% reoxidation is usually observed (Lennon & Williams, 1995). This seeming discrepancy will be explored further below.

Spectra were taken during turnover as shown in Figure 3. The spectrum taken at the end of the dead time (spectrum 2) shows a decrease in extinction at 456 nm, which is accompanied by the formation of a broad band centered at about 550 nm, but which also extends past 700 nm. The spectral changes are consistent with the formation of an NADPH−FAD charge transfer complex (Blankenhorn, 1975), and have been observed in the reaction of thioredoxin reductase with NADPH in the absence of thioredoxin (Massey et al., 1970; Williams et al., 1991; Lennon, 1995). The spectrum at 192 ms (spectrum 3), which is seen in the early part of the turnover portion of the reaction, clearly shows that the flavin has been largely reduced. The long-wavelength region of this spectrum is fairly flat, and does not resemble either the NADPH−FAD or the reduced flavin−$NADP^+$ charge transfer bands (see below). This spectrum is maintained throughout most of turnover. Toward the end of turnover, as the NADPH has been consumed and $NADP^+$ has been produced, the spectrum begins to exhibit the spectral characteristics of a reduced flavin−$NADP^+$ charge transfer complex, as evidenced by an increase in extinction which is maximal near 700 nm (spectrum 4, Figure 3) (Zanetti & Williams, 1967; Blankenhorn, 1975; Massey & Palmer, 1962; Claiborne & Ahmed, 1989). The 2 min spectrum shows the partial reoxidation of the flavin by thioredoxin following turnover (spectrum 5, Figure 3). Note that the extinction at 700 nm has dropped during this reoxidation process. This is due to the loss of reduced flavin, which is necessary to form the reduced flavin−$NADP^+$ charge transfer complex. Note also that the long-wavelength region shows the presence of a new, broad double peak centered at about 610 nm, which is caused by the presence of the neutral flavin semiquinone. It has been previously established that the exposure of partially reduced thioredoxin reductase to light is capable of producing flavin semiquinone quite easily (Zanetti et al., 1968). That this is indeed the case in this experiment is demonstrated by spectrum 6 in Figure 3. This spectrum was obtained by leaving the shutter open on the stopped-flow instrument for 3 min after the 2 min spectrum was taken, thus exposing the partially reduced enzyme to intense white light. This spectrum compares favorably with previously published spectra of flavin semiquinone produced under similar conditions (Zanetti et al., 1968).

As mentioned before, the 192 ms spectrum (spectrum 3) in Figure 3, taken early in turnover, shows a rather featureless band in the long-wavelength region which does not resemble either an NADPH−FAD or a reduced flavin−$NADP^+$ charge transfer complex. The long-wavelength region in the 192 ms turnover spectrum strongly resembles that of fully reduced thioredoxin reductase in the presence of excess NADPH. Under the latter conditions, the enzyme is fully reduced, and the $NADP^+$ produced by reduction of the enzyme has been displaced by the excess NADPH present so that the spectrum represents fully reduced enzyme with NADPH bound (Lennon, 1995). Thus, it appears that the most prevalent enzyme species present during turnover contains essentially fully reduced flavin, and has NADPH

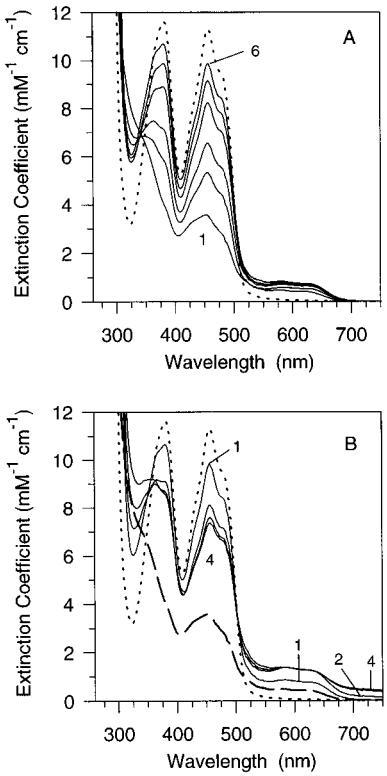


FIGURE 4: Titration of reduced thioredoxin reductase with oxidized thioredoxin and NADP$^+$. The titrations were done in 0.1 M sodium/potassium phosphate, pH 7.6 at 25 °C. Panel A: Titration with oxidized thioredoxin. Initial enzyme concentration was 24.4 μM, reduced with 2.1 equiv of sodium dithionite. Concentrations of thioredoxin are (1) 0 μM, (2) 24.3 μM, (3) 48.0 μM, (4) 116 μM, (5) 199 μM, and (6) 310 μM. The dotted line is a spectrum of fully oxidized enzyme for reference. Panel B: Titration with NADP$^+$. Starting sample is the final sample from panel A, containing 20.5 μM enzyme and 310 μM thioredoxin. Concentrations of NADP$^+$ are (1) 0 μM, (2) 22.3 μM, (3) 88.6 μM, and (4) 164 μM. Note the increase in long-wavelength absorbance (~750 nm) in spectra 2 through 4 due to the formation of the reduced flavin−NADP$^+$ charge transfer complex. For reference, the dashed spectrum is reduced enzyme at the start of the thioredoxin titration shown in panel A, and the dotted spectrum is fully oxidized enzyme.

bound until sufficient NADP$^+$ is produced in turnover to displace the NADPH. As has been shown in other work from this lab, NADPH and NADP$^+$ apparently bind competitively to thioredoxin reductase (Williams, 1976; Lennon, 1995).

Titration of Fully Reduced Enzyme with Thioredoxin and NADP$^+$. The difference in the extent of flavin reoxidation by thioredoxin in the presence and absence of NADP$^+$ (see above) was studied in an equilibrium titration. Enzyme fully reduced with sodium dithionite was titrated with up to 15 equiv of oxidized thioredoxin (Figure 4A). Without correcting for semiquinone formed (evidenced by the broad double peak centered at ~610 nm), the flavin is approximately 85% oxidized at the end of the titration. This mixture of mostly oxidized enzyme and thioredoxin, which contained some reduced thioredoxin, was then titrated with NADP$^+$ (Figure 4B). Despite the fact that an oxidant was being added, and that the extinction in the 340 nm region increased due to the formation of some NADPH, the flavin peak extinction dropped back to about 50% that of oxidized enzyme. This is in good agreement with the levels of flavin reoxidation observed at the end of the turnover experiment (curve 2, Figure 2). This effect is due to a perturbation of the equilibrium between oxidized and reduced enzyme and thioredoxin caused by the binding of NADP$^+$ to the enzyme, and will be clarified in the Discussion. That the enzyme has NADP$^+$ bound is evidenced by the increase in extinction at 700 nm due to the formation of a reduced flavin−NADP$^+$ charge transfer complex (Figure 4B, spectra 1−4). The amount of semiquinone is virtually unchanged between spectra 1 and 4, the increase at 600 and 700 nm being due primarily to the formation of the reduced flavin−NADP$^+$ charge transfer complex.

DISCUSSION

The X-ray crystal structure of thioredoxin reductase shows the redox-active disulfide adjacent to the *re* face of the isoalloxazine ring of FAD. It does not reveal a continuous path for reducing equivalents, in particular from NADPH to FAD or from the nascent dithiol to the bound substrate, thioredoxin (Figure 1, lower left panel). NADPH can be brought adjacent to the *re* face of the flavin ring, and the dithiol can be presented to a potential thioredoxin binding site by the same unimpeded rotation of the pyridine nucleotide domain relative to the FAD binding domain (Waksman et al., 1994) (Figure 1, lower right panel). Thus, the occurrence and reversal of the proposed conformational change enable NADPH and the active site sulfurs to alternately interact with the flavin. It is believed that the enzyme exists in solution in an equilibrium mixture of both conformations (Lennon, 1995), although it is not known what effect the binding of NADPH or enzyme reduction may have on the proposed conformational change. Evidence does exist which suggests that the binding of thioredoxin favors the FR conformation (Wang et al., 1996), probably by binding to the FR conformation and pulling the equilibrium to favor that conformation.

Given these structural features, the most direct pathway for reducing equivalents in turnover would require a ternary complex in which the flavin is reduced by NADPH while thioredoxin is being reduced by the active site dithiol when the enzyme is in the proposed FR conformation. Following the reversal of rotation, the nascent active site disulfide could be reduced by the newly reduced flavin. This would require only one rotation in each direction for each catalytic cycle. We previously showed that complexation of pyridine nucleotide with reduced thioredoxin reductase decreased the rate of reoxidation of the enzyme by thioredoxin to a rate essentially equal to k_{cat}, and this was interpreted as evidence favoring such a ternary complex mechanism (Lennon & Williams, 1995). It was suggested that the actual step limiting the observed reoxidation of enzyme by thioredoxin (as monitored by flavin absorbance) could be either the dithiol/disulfide interchange between reduced enzyme and oxidized thioredoxin or the conformational change discussed above (Lennon & Williams, 1995).

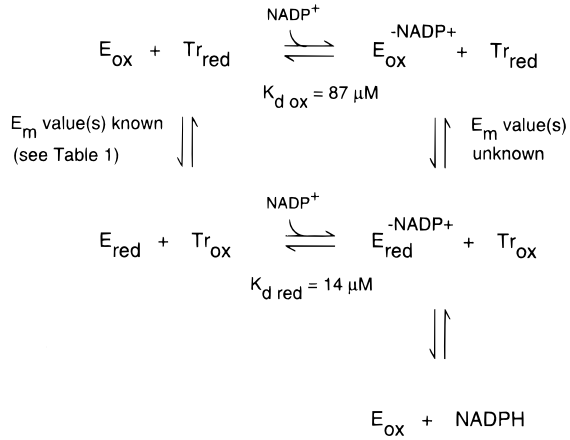
A basic goal of this study was to determine the redox state of thioredoxin reductase during turnover, since the ternary mechanism described above implies that thioredoxin reductase should cycle between the four-electron-reduced and two-electron-reduced states during catalysis. The spectrum observed during turnover in the current work represents an enzyme species which contains largely reduced flavin. Since the midpoint potentials of the flavin and of the redox-active disulfide in thioredoxin reductase are so similar (see Table

1), and if the equilibration of electrons between the two redox centers is rapid so that the redox states are determined by the respective midpoint potentials and not by a kinetic step, then the redox state of the flavin would reflect the redox state of the disulfide/dithiol couple. However, flavin reduction by NADPH and the reduction of thioredoxin by the dithiol of thioredoxin reductase are thought to occur in the postulated FR conformation (Figure 1), while the equilibration of electrons between the flavin and the enzyme disulfide occurs in the FO conformation which is observed in the crystal structure (Waksman et al., 1994). Thus, following the reduction of flavin, the conformational change precedes the equilibration of electrons between the two redox centers. Kinetic modeling of the reductive half-reaction of thioredoxin reductase (Lennon, 1995) has indicated that the rate of this proposed conformational change is slower than the rate of flavin reduction at high concentrations of NADPH (flavin reduction = 69 s^{-1} at 1 °C, 590 s^{-1} at 25 °C, cf. k_{cat} = 33 s^{-1} at 25 °C). If the rate of the proposed conformational change is indeed slow, then it is possible that the distribution of electrons between the flavin and the enzyme disulfide/dithiol couple is not determined solely by the midpoint potentials, but also by the rate of a slow step such as the conformational change. In this case, the reduction state of the flavin during turnover will not necessarily reflect the reduction state of the disulfide/dithiol couple, and so the total redox state of the enzyme during turnover cannot be determined solely from the flavin absorbance. The evidence which suggested this comes entirely from modeling, not from a direct measurement of the rate of electron equilibration between redox centers. Further study will be required to accurately determine whether the conformational change and the electron equilibration between redox centers is fast or slow. However, two further points of evidence which are directly relevant to the question of the redox state of the disulfide will be described.

First, the midpoint potentials of the various couples involved in the thioredoxin reductase reaction provide support for the idea that thioredoxin reductase cycles between the four-electron-reduced and two-electron-reduced states. It is the disulfide/dithiol couples in thioredoxin reductase which are involved in reducing thioredoxin. As shown in Table 1, the midpoint potential for the disulfide/dithiol couple in enzyme containing reduced flavin is 17 mV lower than that in enzyme containing oxidized flavin, making the four-electron-reduced species (−287 mV) a more efficient reductant for thioredoxin (−282 mV). Thus, the two-electron-reduced species interacting with NADPH is the FAD/$FADH_2$ couple in enzyme containing an active site dithiol. While its midpoint potential is 17 mV lower than that of the FAD/$FADH_2$ couple in enzyme containing an active site disulfide, it is still 33 mV higher than the potential of NADPH. Thus, NADPH is an adequate reductant for regenerating four-electron-reduced enzyme.

Second, support for the conclusion that thioredoxin reductase cycles between the two-electron- and four-electron-reduced states comes from the spectral properties of the enzyme species present prior to what is thought to be the rate-limiting step in catalysis. The spectrum observed during the turnover portion of the enzyme-monitored turnover experiment (spectrum 3, Figure 3) should largely reflect the spectral characteristics of the enzyme species which precedes the rate-limiting step in catalysis. We concluded in earlier work that the rate-limiting step in catalysis involves the reaction between a reduced enzyme−pyridine nucleotide complex and oxidized thioredoxin, and may be limited either by the rate of dithiol−disulfide interchange or by the rate of the conformational change proposed by Waksman et al. (1994) which allows the oxidation of flavin by the nascent enzyme disulfide (Lennon & Williams, 1995). Since the proposed limiting step is one which involves the oxidation of the enzyme, then the enzyme species immediately prior to the rate-limiting step may represent the most reduced form of the enzyme present during catalysis. Since this species is interacting with thioredoxin, it must contain a dithiol, at least transiently, and since it contains reduced flavin (spectrum 3, Figure 3), it appears that the species interacting with thioredoxin in the rate-limiting step is four-electron-reduced enzyme. This supports the conclusion that turnover occurs between the four-electron-reduced and two-electron-reduced states.

Scheme 1: Proposed Mechanism for the Decrease in Flavin Extinction Observed upon Addition of $NADP^+$ to a Mixture of Oxidized and Reduced Thioredoxin Reductase and Thioredoxin[a]



[a] Abbreviations: E_{ox}, oxidized enzyme; E_{red}, reduced enzyme; Tr_{ox}, oxidized thioredoxin; Tr_{red}, reduced thioredoxin. The K_d values shown for $NADP^+$ are from Lennon and Williams (1995).

The spectrum of thioredoxin reductase during turnover not only shows that the enzyme contains reduced flavin but also indicates that pyridine nucleotide is bound. In addition, it was observed in the enzyme-monitored turnover experiment that the presence of $NADP^+$ decreases the degree to which thioredoxin is able to reoxidize thioredoxin reductase. We have presented evidence that a ternary complex of reduced enzyme, pyridine nucleotide, and oxidized thioredoxin is catalytically relevant (Lennon & Williams, 1995). This recurring theme of significant effects of pyridine nucleotides on catalysis (Matthews & Williams, 1976; Matthews et al., 1976, 1979) is further illustrated by the experiment (Figure 4B) in which the titration of reduced enzyme with thioredoxin was followed by the addition of $NADP^+$. It has been shown that $NADP^+$ binds to fully reduced thioredoxin reductase more tightly than to the oxidized form ($K_{d\ red}$ = 14.0 ± 2.2 μM, $K_{d\ ox}$ = 87.1 ± 2.5 μM) (Lennon & Williams, 1995). At the end of the titration shown in Figure 4A, the reaction mixture contained oxidized and reduced thioredoxin as well as oxidized and reduced enzyme, and so a redox equilibrium existed between the enzyme and thioredoxin (first column, Scheme 1). Since $NADP^+$ binds to the reduced enzyme over 6-fold more tightly than to the oxidized enzyme, as $NADP^+$

is added it perturbs the equilibrium between the two enzyme redox forms to favor the reduced form (center species, right hand column, Scheme 1). This has the effect of raising the midpoint potentials of the enzyme. Electrons were passed on from the flavin to the $NADP^+$ in this experiment, as evidenced by the production of NADPH observed in Figure 4B (bottom species, right hand column, Scheme 1). However, it appears that the reduction of $NADP^+$ by the flavin is less favorable than the proposed perturbation of the thioredoxin reductase−thioredoxin redox equilibrium by $NADP^+$. Thus, electrons accumulate on the flavin. In the enzyme-monitored turnover experiment, there is an additional reason for the low degree of reoxidation of enzyme by thioredoxin. Not only has the presence of $NADP^+$ caused an apparent increase in the enzyme midpoint potentials, but the reduced thioredoxin produced during turnover is making the equilibrium for the reoxidation of enzyme by thioredoxin even less favorable.

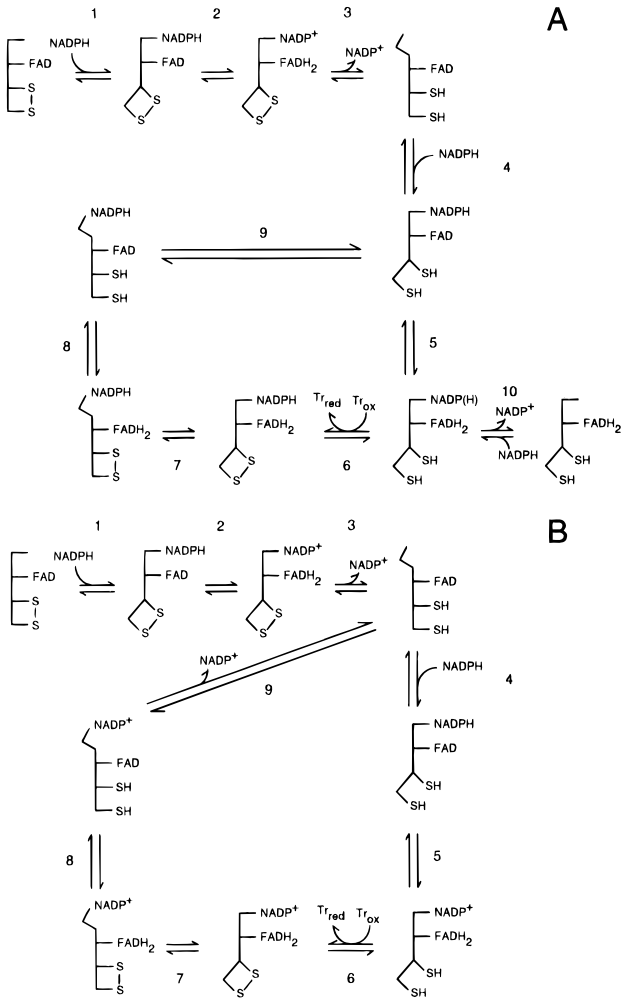
This perturbation of enzyme midpoint potentials by the differential binding of $NADP^+$ to oxidized and reduced enzyme is similar to that reported for adrenodoxin reductase (Lambeth & Kamin, 1976) and for lipoamide dehydrogenase (Hopkins & Williams, 1995). Lambeth and Kamin (1976) used the concept of a thermodynamic box, and showed that if three of the four constants (two K_d values and two midpoint potentials) describing the box are known the value of the fourth, unknown, constant can be calculated. Equation 7 from Lambeth and Kamin (1976) was used (eq 1):

$$K_r = K_o \times 10^{-(nF\Delta E_m/2.3RT)} \quad (1)$$

where $K_r = K_d$ for $NADP^+$ with reduced enzyme, $K_o = K_d$ for $NADP^+$ with oxidized enzyme, and ΔE_m is the change in the enzyme midpoint potential in the presence and absence of $NADP^+$. In Scheme 1 for thioredoxin reductase, the values for the K_d of $NADP^+$ with oxidized and reduced enzyme are known, as well as the midpoint potentials of the free enzyme. Ignoring the step leading to the formation of NADPH as being disfavored, it can be calculated that the midpoint potentials in thioredoxin reductase increase by 23 mV upon binding $NADP^+$. A strong cautionary statement regarding this calculation is in order. In the experiment of Figure 4 with thioredoxin reductase, the sample contains thioredoxin, which is able to bind to the enzyme. It is reasonable to assume that the binding affinity for thioredoxin to the oxidized and reduced enzymes is different, and so the presence of thioredoxin will probably introduce further perturbations in the midpoint potentials of thioredoxin reductase. Thus, the calculated increase in midpoint potential described here is only intended to be illustrative of the fact that when a ligand (in this case $NADP^+$) binds more tightly to the reduced form of an enzyme than to the oxidized, it increases the midpoint potential of the enzyme. It should not be used to calculate a specific final value for the enzyme midpoint potential(s) upon binding $NADP^+$ when thioredoxin is present.

The binding of $NADP^+$ may play a regulatory role in thioredoxin reductase activity, since it is only when the ratio of $NADP^+$ to NADPH is high that the amount of $NADP^+$ bound will be significant. When this occurs, the rise in midpoint potentials would make the enzyme a poorer reductant for the substrate thioredoxin, thus making the reaction less favorable and preventing the consumption of

Scheme 2: Proposed Mechanisms for Turnover of *E. coli* Thioredoxin Reductase[a]



[a] The schematic backbone diagrams indicate whether the enzyme is in the FO (active site sulfurs near the flavin) or the FR (pyridine nucleotide binding site near the flavin) conformation proposed by Waksman et al. (1994). The starting enzyme species does not display either of these conformations explicitly, since it is not yet known which conformation predominates in the oxidized enzyme. Indeed, it is possible that in all the enzyme species shown the enzyme actually exists in both conformations, but only those conformations which are productive in terms of the chemistry occurring at each step are displayed. While the binding, release, or presence of pyridine nucleotide has been shown as accompanying the rotation events, no evidence yet exists to indicate how pyridine nucleotide or the redox state of the enzyme affects the conformational change during turnover. Panel A: Proposed mechanism for early turnover, where the concentration of NADPH is high. Panel B: Proposed mechanism for late turnover, where the concentration of NADPH is low.

further NADPH. It is not useful to speculate further on this point since, as mentioned above, the effect of the binding of thioredoxin on the enzyme midpoint potentials is not known.

The observations and conclusions from this work and previous work (Lennon & Williams, 1995; Williams, 1995) have been combined to produce the general mechanisms for turnover shown in Scheme 2. Scheme 2A shows a mechanism for turnover early in the reaction, when the concentration of NADPH is still high. Steps 1−5 comprise the reductive half-reaction (i.e., the reduction of oxidized enzyme by 2 equiv of NADPH) (Zanetti & Williams, 1967; Williams et al., 1991; Lennon, 1995). This produces a four-electron-reduced enzyme species which has $NADP^+$ bound. Once step 5 has been completed, since NADPH levels are high,

equilibrium 10 will exchange $NADP^+$ and NADPH, so that the species following step 5 will then primarily have NADPH bound. This conclusion is supported by the study of the reductive half-reaction, which indicates that the replacement of $NADP^+$ by NADPH on reduced enzyme is facile (Lennon, 1995). This is followed by the reduction of thioredoxin by the enzyme dithiol in step 6. A conformational change follows in step 7, which allows the nascent enzyme disulfide to approach the reduced flavin. This in turn allows the possibly rapid equilibration of electrons between the flavin and the active site sulfurs in step 8, followed by a second conformational change in step 9 to allow the reduction of the now oxidized flavin by the NADPH already bound to the enzyme. Thus, turnover proceeds through steps 5 through 9, with equilibrium 10 replacing the $NADP^+$ produced by flavin reduction with NADPH for the next round of flavin reduction.

Scheme 2B shows a mechanism for turnover later in the reaction, as NADPH is consumed and $NADP^+$ levels rise. Steps 1−5 of this mechanism are identical to those in Scheme 2A, but due to the low levels of NADPH, equilibrium 10 from Scheme 2A is eliminated, which causes the product of step 5 to retain the $NADP^+$ which is already bound to it following flavin reduction. Steps 6 through 8, i.e., reduction of thioredoxin, a conformational change, and equilibration of electrons between the flavin and the active site sulfurs, are the same as in Scheme 2A except that $NADP^+$ is bound instead of NADPH. This change in the mechanism was suggested by the fact that the enzyme spectrum changes from that of reduced flavin with NADPH bound early in turnover to that of reduced flavin with $NADP^+$ bound late in turnover. $NADP^+$ is released in step 9 in preparation for the binding of NADPH in step 4. Thus, turnover proceeds through steps 4 through 9 in Scheme 2B when the concentration of NADPH is low.

The placement of the step involving the reduction of thioredoxin (step 6, Scheme 2A,B) such that it involves the reaction with four-electron-reduced enzyme instead of the two-electron-reduced enzyme is based on the evidence provided by the midpoint potentials discussed above, as well as on the spectral properties of the enzyme observed during turnover and what is believed to be the rate-limiting step in catalysis. As was mentioned earlier, it is believed that the rate-limiting step either is dithiol−disulfide interchange between oxidized thioredoxin and a reduced enzyme−pyridine nucleotide complex (step 6, Scheme 2A,B) or is the postulated conformational change which allows the oxidation of flavin by the nascent enzyme disulfide following the reduction of thioredoxin (step 7, Scheme 2A,B) (Lennon & Williams, 1995). Regardless of whether the rate-limiting step is dithiol−disulfide interchange (step 6) or the conformational change (step 7), the enzyme species preceding these steps contain reduced flavin and pyridine nucleotide. Thus, Scheme 2A and Scheme 2B agree well with the evidence in Figure 3. If thioredoxin reductase were cycling between the two-electron-reduced and oxidized enzyme forms, then thioredoxin would have to react with the two-electron-reduced enzyme, which would necessarily contain FAD and a dithiol. In this case, the rate-limiting step would be preceded by an enzyme form containing oxidized flavin, which is contrary to the evidence in Figure 3.

There is an important caveat in the interpretation of Scheme 2. While we have shown the conformational change events as occurring either after $NADP^+$ release, after NADPH binding, or simply with pyridine nucleotide bound, it should be stressed again that it is not currently known what truly causes the postulated conformational change to occur. Indeed, the starting enzyme species in Scheme 2 does not display either conformation explicitly, since it is not yet known which conformation is predominant in the oxidized enzyme. While the binding of thioredoxin appears to favor the conformational change (Wang et al., 1996), it clearly must occur in the absence of thioredoxin as well, in order to produce four-electron-reduced enzyme in the reaction with NADPH. Thus, the redox state of either the flavin or the active site disulfide/dithiol couple, as well as the binding or release of pyridine nucleotides, has the potential to affect the conformational change. Indeed, it is possible that in all the enzyme species shown in Scheme 2 the enzyme actually exists in both the FR and FO conformations, but only those conformations which are productive in terms of the chemistry occurring at each step are displayed.

As discussed and laid aside (Lennon & Williams, 1995), a ping-pong mechanism can be envisioned for thioredoxin reductase. In the current work, this would necessitate that Scheme 2A be modified to allow the reaction of thioredoxin with free reduced enzyme (i.e., the product to the right of step 10). We have published evidence that this reaction can occur, but that it is not catalytically relevant (Lennon & Williams, 1995). Moreover, turnover with pyridine nucleotide bound (Figure 3) requires that NADP(H) be bound to the enzyme species prior to the rate-limiting step of the reaction. If step 6 in Scheme 2A is limiting, then NADP(H) must rebind immediately, before the reaction with thioredoxin. If step 7 is limiting, then NADP(H) must rebind to the enzyme immediately after step 6, and before the conformational change, in order to agree with the results in Figure 3. We believe that these are unnecessary complications of the mechanism. It should also be noted that the ternary complex mechanism proposed here and in Lennon and Williams (1995) is intuitively more efficient than a ping-pong mechanism. In a ternary complex, the flavin can interact with the pyridine nucleotide at the same time that thioredoxin is being reduced by the enzyme active site dithiol (Figure 1, bottom right panel).

This work indicates that thioredoxin reductase proceeds through catalysis via a four-electron- to two-electron- to four-electron-reduced cycle, and that it does so with NADP(H) bound. This is in agreement with previously published work (Lennon & Williams, 1995), and lends strong support to the proposal that thioredoxin reductase utilizes a ternary complex mechanism in catalysis. A ternary complex provides the simplest pathway for reducing equivalents where sequential juxtaposition of the various redox components requires rotation of one domain relative to another.

ACKNOWLEDGMENT

We thank Dr. Marian T. Stankovich of the Department of Chemistry, University of Minnesota, and Dr. Scott B. Mulrooney of the Department of Biological Chemistry, The University of Michigan, for their critical reading of this paper.

REFERENCES

Blankenhorn, G. (1975) *Eur. J. Biochem. 50*, 351−356.
Claiborne, A., & Ahmed, S. A. (1989) *J. Biol. Chem. 264*, 19864−19870.

Clark, W. M. (1960) in *Oxidation-Reduction Potentials of Organic Systems*, pp 254, 264, 490, and 522, The Williams and Wilkins Company, Baltimore.
Engel, P. C., & Dalziel, K. (1967) *Biochem. J. 105*, 691−695.
Gibson, Q. H., Swoboda, B. E. P., & Massey, V. (1964) *J. Biol. Chem. 239*, 3927−3934.
Holmgren, A. (1968) *Eur. J. Biochem. 6*, 475−484.
Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237−271.
Hopkins, N. H., & Williams, C. H., Jr. (1995) *Biochemistry 34*, 11766−11776.
Krause, G., Lundström, J., Barea, J. L., De la Cuesta, C. P., & Holmgren, A. (1991) *J. Biol. Chem. 266*, 9494−9500.
Lambeth, J. D., & Kamin, H. (1976) *J. Biol. Chem. 251*, 4299−4306.
Lennon, B. W. (1995) Ph.D. Dissertation, The University of Michigan, University Microfilms, Ann Arbor.
Lennon, B. W., & Williams, C. H., Jr. (1995) *Biochemistry 34*, 3670−3677.
Lockridge, O., Massey, V., & Sullivan, P. A. (1972) *J. Biol. Chem. 247*, 8097−8106.
Massey, V., Matthews, R. G., Foust, G. P., Howell, L. G., Williams, C. H., Jr., Zanetti, G., & Ronchi, S. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., Ed.) pp 393−409, Springer-Verlag, Berlin.
Massey, V., & Palmer, G. (1962) *J. Biol. Chem. 237*, 2347−2358.
Matthews, R. G., & Williams, C. H., Jr. (1976) *J. Biol. Chem. 251*, 3956−3964.
Matthews, R. G., Wilkinson, K. D., Ballou, D. P., & Williams, C. H., Jr. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 464−472, Elsevier Scientific, New York.
Matthews, R. G., Ballou, D. P., & Williams, C. H., Jr. (1979) *J. Biol. Chem. 254*, 4974−4981.
Mayhew, S. G. (1978) *Eur. J. Biochem. 85*, 535−547.
Moore, E. C., Reichard, P., & Thelander, L. (1964) *J. Biol. Chem. 239*, 3445−3452.
Mulrooney, S. B., & Williams, C. H., Jr. (1994) *Biochemistry 33*, 3148−3154.
O'Donnell, M. E., & Williams, C. H., Jr. (1983) *J. Biol. Chem. 258*, 13795−13805.
O'Sullivan, W. J. (1969) in *Data for biochemical research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M., Eds.) pp 423−434, Oxford University Press, New York.
Russel, M., & Model, P. (1988) *J. Biol. Chem. 263*, 9015−9019.
Stoll, V. S., & Blanchard, J. S. (1990) *Methods Enzymol. 182*, 24−38.
Waksman, G., Krishna, T. S. R., Williams, C. H., Jr., & Kuriyan, J. (1994) *J. Mol. Biol. 236*, 800−816.
Wang, P.-F., Veine, D. M., Ahn, S. H., & Williams, C. H., Jr. (1996) *Biochemistry* (in press).
Watt, G. D., & Burns, A. (1975) *Biochem. J. 152*, 33−37.
Williams, C. H., Jr. (1976) in *The Enzymes* (Boyer, P. D., Ed.) pp 89−173, Academic Press, New York.
Williams, C. H., Jr. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. III, pp 121−211, CRC Press, Boca Raton, FL.
Williams, C. H., Jr. (1995) *FASEB J. 9*, 1267−1276.
Williams, C. H., Jr., Prongay, A. J., Lennon, B. W., & Kuriyan, J. (1991) in *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 497−504, Walter de Gruyter & Co., Berlin.
Zanetti, G., & Williams, C. H., Jr. (1967) *J. Biol. Chem. 242*, 5232−5236.
Zanetti, G., Williams, C. H., Jr., & Massey, V. (1968) *J. Biol. Chem. 243*, 4013−4019.

BI952521I