Effect of Pyridine Nucleotide on the Oxidative Half-Reaction of *Escherichia coli* Thioredoxin Reductase†

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ABSTRACT: The kinetics of the oxidative half-reaction between reduced thioredoxin reductase and oxidized thioredoxin measured in the presence and absence of pyridine nucleotide show a significant difference in the rates of the main phase of oxidation. When 1 equiv of NADPH is used to partially reduce the enzyme at pH 7.0 or 7.6, the observed rate of the catalytically competent phase of oxidation is essentially equal to k_{cat} at that pH. This is about 50% of the rate of oxidation observed with enzyme fully reduced or partially reduced by the xanthine/xanthine oxidase system or by dithionite. Through the use of the nonreducible analog 3-aminopyridine adenine dinucleotide phosphate we have shown that this decrease in observed rate of oxidation is linked to the concentration of pyridine nucleotide present. This suggests that the complexation of pyridine nucleotides with reduced thioredoxin reductase is able to effect a change in the rate-limiting steps of the oxidation of the enzyme by thioredoxin. This is the case even when substoichiometric quantities of 3-aminopyridine adenine dinucleotide phosphate are present, which predicts that the binding to reduced enzyme is very tight. It is clear that the presence of 1 equiv of $NADP^+$ is sufficient to cause the observed rate for the catalytically competent phase of oxidation to decrease to k_{cat}. Thus, there is compelling evidence for a ternary complex mechanism for thioredoxin reductase.

Thioredoxin reductase (EC 1.6.4.5) is a member of the pyridine nucleotide-disulfide oxidoreductase family, which also includes lipoamide dehydrogenase, glutathione reductase, and mercuric ion reductase (Williams, 1990). The enzyme 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). This enzyme catalyzes the reduction of a small protein (M_r = 11,700), thioredoxin, by NADPH (Moore et al., 1964; Holmgren, 1968). The passage of electrons is from NADPH to 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).

Evidence has been gathered which supports a ping-pong mechanism for both lipoamide dehydrogenase and glutathione reductase (Williams, 1990). While steady-state kinetics with thioredoxin reductase produce a series of parallel lines in Lineweaver−Burk plots which is indicative of a ping-pong catalytic mechanism (Prongay et al., 1989), this is by no means definitive, and considerable detail regarding the mechanism is still lacking. It is also not sufficient to rely on similarity to other family members, given the differences that exist between thioredoxin reductase and the other family members (Williams, 1990). For instance, in human erythrocyte glutathione reductase the pyridine nucleotide binds on the *re* side of the isoalloxazine ring, and dithiol/disulfide interchange takes place on the *si* side (Pai & Schulz, 1983). In thioredoxin reductase, on the other hand, both the redox-active disulfide and the pyridine nucleotide binding site are on the *re* side of the flavin (Kuriyan et al., 1991). This is a direct consequence of the fact that the cysteine residues comprising the active site disulfide are in the pyridine nucleotide domain in thioredoxin reductase but in the FAD domain in the other members of the family (Russel & Model, 1988). It is also significant that, in contrast to other family members, one of the substrates for thioredoxin reductase is itself a protein which is about 1/3 the size of the enzyme monomer.

Earlier work by others reported that when *E. coli* thioredoxin reductase, partially reduced with 1 equiv of NADPH, was used in the oxidative half-reaction with thioredoxin, a monophasic oxidation of flavin with a rate of ~20 s^{-1} was observed independent of the concentration of thioredoxin, indicating that the binding of thioredoxin to the reduced enzyme is tight (Navarro et al., 1991). Since the observed rate of oxidation was essentially equal to k_{cat} under those conditions (23 s^{-1}), it was concluded by those authors that the rate-limiting step in catalysis lies entirely in the oxidative half-reaction and may represent an electron-transfer step within a complex of thioredoxin reductase and thioredoxin. However, since there was a small amount of product from the reductive half-reaction present ($NADP^+$) and the enzyme was at an intermediate redox state, it was possible that the reaction observed in that study did not reflect the isolated oxidative half reaction. In the present work we have studied the oxidative half reaction of thioredoxin reductase from *E. coli* in both partially and fully reduced forms, as well as in the presence and absence of pyridine nucleotides. We present evidence that, while it does not rule out a ping-pong

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mechanism, does suggest that catalysis may proceed via a ternary complex mechanism. Some of this work has been presented in an abbreviated form (Lennon & Williams, 1994).

MATERIALS AND METHODS

Reagents. $NADP^+$ (Sigma grade), NADPH (type III, enzymatically reduced), protocatechuic acid (3,4-dihydroxybenzoic acid) (PCA),[1] xanthine, and bovine liver catalase were all purchased from Sigma. Sodium dithionite was purchased from Fluka Chemical Corp. Benzyl viologen was purchased from Mann Research Laboratories, Inc. Protocatechuate dioxygenase (PCD), lumiflavin-3-acetic acid, and bovine milk xanthine oxidase were the generous gifts of Dr. David P. Ballou (PCD) and Dr. Vincent Massey (lumiflavin-3-acetate and xanthine oxidase) of the Department of Biological Chemistry, University of Michigan. All other reagents and buffers were of the highest quality available.

E. coli Thioredoxin Reductase Purification. Wild-type thioredoxin reductase was grown in a *trxB*$^-$ strain of *E. coli* containing a high copy number plasmid which carried the *trxB* gene and imparted trimethoprim resistance (K1380.pTrR1) (Mulrooney & Williams, 1994). Cells were grown to saturation in Mueller Hinton broth at 37 °C. Cells were harvested and lysed by sonication in 20 mM Tris, 0.3 mM EDTA, pH 7.6, and 0.1 mM phenylmethanesulfonyl fluoride. The suspension was brought to 2% (w/v) streptomycin sulfate and stirred at 4 °C for 1 h before it was centrifuged at 38800*g* for 15 min and then at 196400*g* for 1 h. The supernatant was dialyzed vs 4 L of the same buffer at 4 °C overnight before it was loaded onto a 5 × 9.9 cm Pharmacia Q Sepharose Fast Flow column equilibrated in 20 mM Tris and 0.3 mM EDTA, pH 7.6, at room temperature. The enzyme was eluted with a step gradient of 0.1 M NaCl increments, with thioredoxin reductase eluting at about 0.6 M NaCl. The main peak was pooled and dialyzed vs 4 L of 10 mM Na/K phosphate and 0.3 mM EDTA, pH 7.6, at 4 °C overnight. The sample was applied to a 2 × 19.5 cm Pharmacia 2′, 5′-ADP Sepharose 4B column equilibrated in the same buffer and washed with 0.2 M NaCl steps until thioredoxin reductase eluted at 0.8 M NaCl. Enzyme was precipitated by the addition of 80% ammonium sulfate and was stored at 4 °C. The enzyme was >99% pure as determined by SDS−PAGE.

Thioredoxin Purification. Thioredoxin was purified from a strain of *E. coli* (MG1655.pDL59) generously provided by LeMaster and Richards (LeMaster & Richards, 1988). The plasmid pDL59 contains the genes for both *E. coli* (*trxA*) and bacteriophage T4 (*nrdC*) thioredoxins, and it also confers ampicillin resistance. A 200-mL culture was grown overnight at 37 °C in TB medium and 50 μg/mL ampicillin in a shaking incubator. This was used as an inoculum for 2 L of the same medium in a Lab-Line Hi-Density fermentor. The cells were grown at 37 °C to an OD_{600} of 5, the temperature was raised to 42 °C, and the growth continued for 3 h before harvesting. Yield was about 80 g wet weight of cells.

The purification was a modification of the method of Laurent et al. (1964). Cell lysis through loading of the Q Sepharose Fast Flow column was the same as for the purification of thioredoxin reductase described above. Thioredoxin was eluted from the Q Sepharose with a linear gradient of 28 to 140 mM NaCl over 7.2 column volumes. T4 thioredoxin elutes before the *E. coli* protein, which is centered at ~130 mM NaCl. The *E. coli* material was pooled, and the protein was precipitated by the addition of 80% ammonium sulfate. The precipitate either was dialyzed vs 2 L of 50 mM Na/K phosphate and 0.3 mM EDTA, pH 7.6, overnight at 4 °C or was simply dissolved in a small volume of the same buffer before application to a 2.5 × 35 cm Sephadex G-50 column and elution. The thioredoxin peak is centered at about 124 mL. This peak was dialyzed exhaustively against deionized water at 4 °C before being lyophilized and stored over desiccant at −20 °C. Purity was determined to be >99% using SDS−PAGE.

Anaerobiosis. Thioredoxin reductase and thioredoxin samples were made anaerobic in tonometers by 10 cycles of evacuation followed by flushing with ultrapure (99.999%) nitrogen which had been passed over an R & D Separations OT3-4 oxygen trap. This procedure was performed on ice to minimize volume loss due to evaporation. PCA (80 μM) and 0.04 unit/mL PCD were added to all thioredoxin samples to remove trace oxygen. Thioredoxin reductase samples also contained the PCA/PCD system except those which were reduced with the xanthine/xanthine oxidase/benzyl viologen system (see below). In all cases the enzyme was scrupulously protected from light during anaerobiosis and reduction.

Reduction of Thioredoxin Reductase. Three methods of reduction were used. Sodium dithionite solutions were prepared anaerobically in 50 mM sodium pyrophosphate buffer, pH 8.90, and were quantified by titration of lumiflavin-3-acetic acid ($\epsilon_{443} = 12\,400\ M^{-1}\ cm^{-1}$). Thioredoxin reductase, made anaerobic as described above in the presence of the PCA/PCD system, was titrated with dithionite added from a Hamilton gastight syringe. No redox mediator was used.

Reduction of flavoenzymes can also be achieved by the use of a system containing xanthine, xanthine oxidase, and a redox mediator (Massey, 1991). Thioredoxin reductase was made anaerobic as described above, but in the presence of 300−350 μM xanthine and 1−2 μM benzyl viologen. After 10 cycles of vacuum and nitrogen flushing, xanthine oxidase and catalase were added from a side arm for final concentrations of 50 nM xanthine oxidase and 100 units/mL catalase. The reaction was allowed to proceed to completion over several hours at room temperature. Full reduction was confirmed by the appearance of the benzyl viologen radical peaks at 399 and 604 nm. In the $AADP^+$ premixing experiments (see below), enzyme was fully reduced by X/XO/BV, and then anaerobic $AADP^+$ was added from a Hamilton gastight syringe.

Reduction of thioredoxin reductase by 1 equiv of NADPH was achieved by making the enzyme anaerobic in the presence of the PCA/PCD system and then tipping in the NADPH from a side arm. Despite the precautions taken to remove oxygen, the enzyme formed a total of ~30% one-electron and three-electron semiquinone species, presumably by reaction with residual oxygen. Navarro et al. (1991) noted that the spectrum of their starting enzyme was similar to that of Williams (1976), Figure 4, p 99. The referenced figure shows no evidence of semiquinone even though that experiment was carried out under conditions similar to those

[1] Abbreviations: PCA, protocatechuic acid (3,4-dihydroxybenzoic acid); PCD, protocatechuic acid dioxygenase; X/XO/BV, xanthine/xanthine oxidase and benzyl viologen reduction system; $AADP^+$, 3-aminopyridine adenine dinucleotide phosphate.

used by Navarro et al. (1991) and in the present study. However, in the experiment in Williams (1976), additions were made without significant pause. We have repeated the experiment which produced Figure 4 in Williams (1976) and find that semiquinone develops within minutes after each addition up to 1 equiv of NADPH provided anaerobiosis is not perfect. Thus, in the time taken to load the reduced enzyme into the stopped-flow apparatus significant semiquinone forms since anaerobiosis is very difficult to maintain under these conditions.

Stopped-Flow Spectrophotometry. Our stopped-flow instrument was designed and built by Dr. David P. Ballou (Department of Biological Chemistry, University of Michigan) and L. David Arscott (VA Medical Center, Ann Arbor, MI). A solution of 80 μM PCA was made anaerobic by bubbling with ultrapure nitrogen for 20 min before 0.04 u/mL PCD was added. This solution was loaded into the stopped-flow instrument at least 4 h prior to the experiment to make the instrument anaerobic. The delivery syringes and mixing block (path length = 2 cm) were immersed in a temperature-controlled water bath which was bubbled with nitrogen in order to mix the water and to make the water somewhat anaerobic. Absorbance changes were detected by shining white light on the sample and then splitting the transmitted light through two optical cables. One cable was connected to a Tracor-Northern TN-6500 rapid scan spectrophotometer which utilizes a photodiode array, enabling the collection of spectra at 5.42-ms intervals. The other cable was connected to a monochromator for continuous monitoring of a wavelength of interest. The monochromatic light was observed with a photomultiplier tube, and data were collected utilizing software written by L. David Arscott (VA Medical Center, Ann Arbor, MI) in the Asyst language (Asyst Software Technologies, Inc.) on a Gateway 2000 486DX running at 33 MHz via a DT2801-A card (Data Translation, Inc.). Kinetic traces were fit by consecutive exponential functions using Program A (developed by Chung-Yen Chiu, Ron Chang, and Joel Dinverno under the direction of David P. Ballou, University of Michigan) which utilizes the Marquardt algorithm (Bevington, 1969). The fitted curves were extrapolated back for the 4-ms dead time of the stopped-flow spectrophotometer in order to more accurately determine the amplitudes of the phases.

The oxidative half-reaction was studied by placing the reduced thioredoxin reductase sample in one syringe and anaerobic thioredoxin samples in the other syringe. The two were mixed rapidly, and the oxidation of flavin was monitored at 456 nm via the monochromator. Experiments were done in 0.1 M Na/K phosphate (pH 7.6) or 20 mM potassium phosphate (pH 7.0) at 25 °C. Enzyme concentrations were 7.5−10 μM after mixing, and concentrations of thioredoxin ranged from 20 to 300 μM after mixing as determined by ϵ_{280} = 13 700 M^{-1} cm^{-1} (Holmgren & Reichard, 1967). Each reaction was repeated 3−5 times, and the fits resulting from each curve were averaged for the final value. Most of the kinetic traces exhibited an early fast decrease in absorbance which was attributed to mixing of the concentrated solutions of thioredoxin with the enzyme solution. The decrease was included in the computer fits to compensate for its influence on the following phases, but it was not regarded as an actual phase of the oxidative half-reaction. The decrease is best seen in the inset to Figure 1 in which the x-axis is a linear scale and the first 50 ms has been expanded. This allows the calculation of the real starting point of the reaction being measured, shown in Figure 1 as a square on the y-axis. Since the starting absorbance of the reduced enzyme is known (circle on the y-axis), an actual phase occurring in the dead time is evident. Given that thioredoxin must bind, undergo dithiol/disulfide interchange, and be released before flavin oxidation can be observed (see Discussion), the early portion of the kinetic trace may contain a lag phase as well as the mixing artifact.

In the experiment where $AADP^+$ was premixed with reduced enzyme, the oxidative half-reaction was observed for 5 s rather than the 0.8 s observed in the other experiments. As a result, a fourth phase appeared in these experiments which had not been previously observed. This phase had an observed rate of 0.38 ± 0.38 s^{-1} and varied in neither rate nor amplitude with increasing concentration of $AADP^+$. Since a fourth phase could also be fit to the data in the control (no $AADP^+$ added), this phase was not caused by the presence of $AADP^+$, but rather was a result of the longer observation time. This phase was not considered relevant to catalysis. Note that data could not be reliably analyzed in the presence of greater than 1 equiv of $AADP^+$ because reduced thioredoxin reductase began to precipitate.

The amplitude of the dead time phase appeared to decrease with increasing concentration of $AADP^+$, suggesting that the presence of pyridine nucleotide affects the amplitude of the dead time phase. In addition, the amplitude of the second phase decreased and the amplitude of the third phase increased with increasing concentration of $AADP^+$. The observed rate of the slower phase, k_3, appeared to increase slightly with increasing concentration of $AADP^+$, and at 11.2 ± 2.9 s^{-1} was faster than was observed in the other experiments shown in Table 1.

Determination of Binding Affinities. To determine the binding affinities of $NADP^+$ and $AADP^+$ to oxidized thioredoxin reductase, 1 mL of ~40 μM thioredoxin reductase in 0.1 M Na/K phosphate, pH 7.6, was placed in the sample position in a Perkin-Elmer Lambda 6 UV-vis spectrophotometer. A cuvette with an identical volume of the same buffer was placed in the reference position. Aliquots of $NADP^+$ or $AADP^+$ were added to both cuvettes, and spectra were taken after each addition. The spectra were converted to extinction, and difference spectra were calculated. Differences in extinction from a positive peak to an adjacent negative peak in the difference spectra were measured to compensate for any baseline shifts. These data were plotted as a double reciprocal. As expected for a K_d in the micromolar range, this plot was nonlinear at lower concentrations of ligand, but at higher concentrations where [ligand] > [enzyme] the plots became linear, and this linear portion was extrapolated to the y-axis to obtain an end point to the titration. The endpoints obtained this way were set equal to the total change in absorbance for 100% enzyme−ligand complex, and this was used to calculate the concentration of enzyme−ligand complex at each point during the titration, including points where the concentration of ligand was not in great excess over enzyme. The K_d for each ligand to oxidized thioredoxin reductase was calculated by using this information and the law of mass action.

Note that when 41 μM thioredoxin reductase reduced with 3.6 equiv of dithionite was titrated with anaerobic $AADP^+$ to determine the K_d of $AADP^+$ with reduced enzyme, there were significant spectral changes. However, at greater than

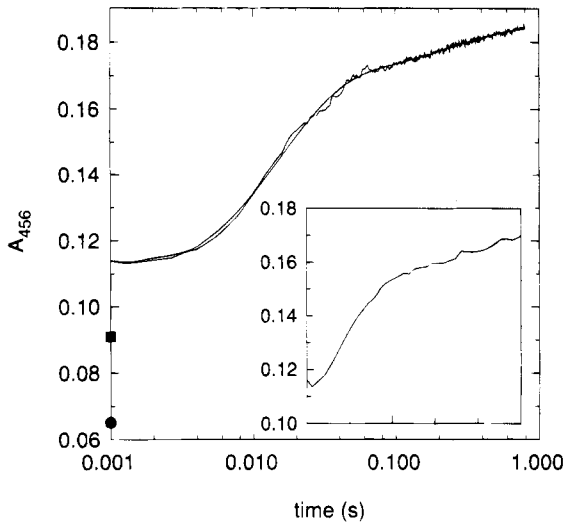

FIGURE 1: Kinetics of oxidation of thioredoxin reductase by thioredoxin. Enzyme was reduced by X/XO/BV and then rapidly mixed with oxidized thioredoxin at pH 7.6. Concentrations were 9.9 μM enzyme and 202 μM thioredoxin after mixing. The smooth curve represents the data fitted by assuming three exponentials where the first exponential is a correction for the mixing artifact visible as a decrease in absorbance prior to the reaction (see the inset). The starting absorbance of the reduced enzyme is indicated by the filled circle, and the starting absorbance of the major phase of oxidation (phase 2, corrected as described in Materials and Methods) is indicated by the filled square. Inset: The first 50 ms of the reaction plotted on a linear time scale to show the mixing artifact.

32 μM $AADP^+$ the enzyme began to precipitate, much as was seen in the $AADP^+$ premixing experiments described above. This made it impossible to determine an end point to the titration, and so no binding constant was obtained.

In determining the K_d of $NADP^+$ to reduced thioredoxin reductase, enzyme was reduced by X/XO/BV, but it was necessary to complete the reduction by the addition of 0.3 equiv of sodium dithionite. The reduced enzyme was rapidly mixed with $NADP^+$ in a Kinetic Instruments Inc. stopped-flow instrument (path length = 1 cm) under the same conditions as the oxidative half-reaction with thioredoxin. The formation of the $NADP^+$–enzyme complex in the dead time was followed by observing the absorbance of the reduced flavin–$NADP^+$ charge-transfer complex at 690 nm. The plot of $1/\Delta A_{690}$ vs $1/[NADP^+]$ was analyzed as described above to obtain the K_d for $NADP^+$ with reduced thioredoxin reductase.

RESULTS

The oxidative half-reaction was observed at pH 7.6 using thioredoxin reductase which was fully reduced either with 2.1 equiv of sodium dithionite or with the X/XO/BV system. These methods of reduction were used so that no material from the reductive half-reaction would be present (i.e., NADP(H)). The two methods of reduction produced comparable results. The reaction showed triphasic kinetics at 456 nm (Figure 1), consisting of an increase complete in the dead time phase (first phase, k_1) followed by two more phases with the observed rate constants shown in the first two lines of Table 1. The rate of the second phase (observed flavin oxidation, k_2) was essentially independent of the concentration of thioredoxin and contained the majority of the change in absorbance (Table 2). Since k_{cat} under these conditions is 33 s^{-1} (Williams, 1976), this second phase represents a catalytically competent step in the oxidative half-reaction.

Table 1: Observed Rates for the Oxidative Half-Reaction of Thioredoxin Reductase[a]

reductant	pH	observed rates (s^{-1}) k_2	k_3
2.1 equiv dithionite	7.6	76.1 ± 17.4	2.2 ± 1.4
X/XO/BV	7.6	70.2 ± 6.1	3.9 ± 2.1
1 equiv NADPH	7.0	23.1 ± 7.7[b]	4.9 ± 0.9
1 equiv NADPH	7.6	35.8 ± 6.2[c]	3.4 ± 1.2
1.2 equiv dithionite	7.6	80.4 ± 8.8	1.0 ± 1.0

[a] Values are averages of rates at 25 °C with ≥5-fold excess thioredoxin. [b] k_{cat} at pH 7.0 is 23 s^{-1} (Navarro et al., 1991). [c] k_{cat} at pH 7.6 is 33 s^{-1} (Williams, 1976).

Table 2: Absorbance Changes for the Oxidative Half-Reaction of Thioredoxin Reductase at the Highest Concentration of Thioredoxin Used, 25 °C.

reductant	pH	[Tr] after mixing (μM)	$\Delta\epsilon_{456}$ (M^{-1} cm^{-1}) phase 1	phase 2	phase 3
2.1 equiv dithionite	7.6	114.5	ND[a]	2157 ± 135	1410 ± 833
X/XO/BV	7.6	202.0	1416 ± 183	4677 ± 358	942 ± 97
1 equiv NADPH	7.0	148.1	257 ± 75	1002 ± 252	1968 ± 288
1 equiv NADPH	7.6	302.5	692 ± 148	1456 ± 162	505 ± 99
1.2 equiv dithionite	7.6	304.7	495 ± 294	1124 ± 221	854 ± 48

[a] ND, not determined.

The fact that the first phase was complete in the dead time suggests that it represents the binding of thioredoxin to the enzyme, where the binding event is able to perturb the spectral properties of the flavin. By the end of the dead time, there were significant amounts of enzyme present which had been oxidized during the major phase of oxidation (phase 2), so the spectral changes caused by binding of thioredoxin are not easily distinguishable from changes caused by oxidation. Mixing artifacts caused by the high concentrations of thioredoxin used made the accurate determination of the amplitude of the dead time phase difficult, and quantitation was not attempted with the sodium dithionite reduced enzyme. However, the amplitude of the dead time phase appeared to increase slightly with increasing concentration of thioredoxin, which supports the conclusion that it represents binding of thioredoxin. Finally, the rate of the third phase of oxidation was also independent of the concentration of thioredoxin, contained a smaller amount of $\Delta\epsilon_{456}$ than the second phase (Table 2), and was too slow to be catalytically significant. The third phase in all the experiments was too slow to be catalytically competent. This phase may represent the establishment of some thermodynamically favorable equilibrium once the kinetic steps have reached completion. The behavior of this phase in the presence of $AADP^+$ (see Materials and Methods) shows that pyridine nucleotide has an effect on it.

The phase following binding, k_2, probably represents a process of oxidation similar to that observed by Navarro et al. (1991), but the rate observed here is approximately 3-fold larger than that of the earlier study. The kinetics of the oxidative half-reaction have therefore been measured under conditions duplicating those of Navarro et al. (1991) as nearly as possible, i.e., using thioredoxin reductase which had been reduced with 1 equiv of NADPH at pH 7.0 (20 mM potassium phosphate), but also at pH 7.6 (0.1 M Na/K

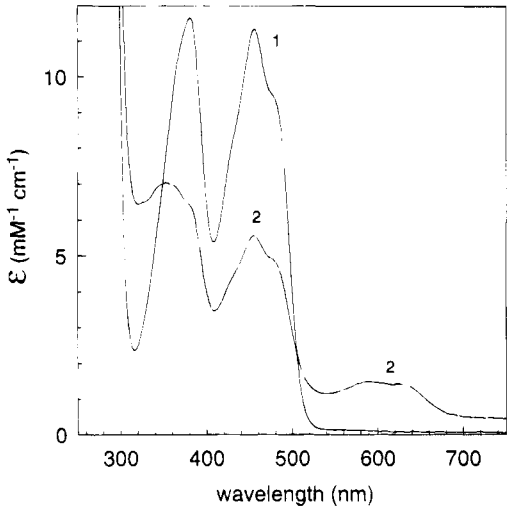

FIGURE 2: Partial reduction of thioredoxin reductase by NADPH in preparation for the oxidative half-reaction. Curve 1, oxidized enzyme at pH 7.6; curve 2, 175 min after the addition of 1 equiv of NADPH.

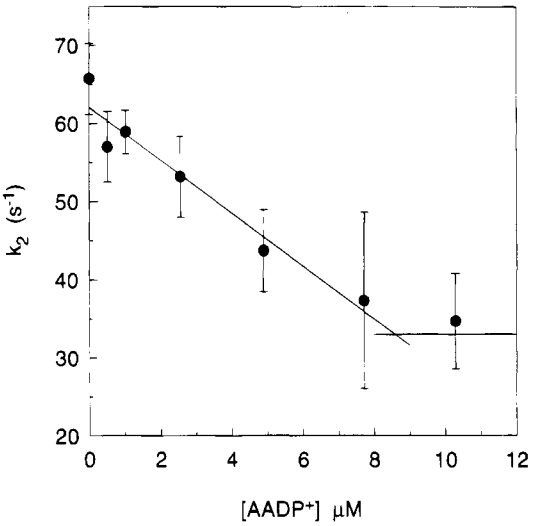

FIGURE 3: Dependence of the observed rate constant, k_2, in the oxidative half reaction on the concentration of AADP$^+$. Final conditions were 10.3 μM thioredoxin reductase and 205 μM thioredoxin in 0.1 M Na/K phosphate, pH 7.6. The sloped line is a linear regression of the first six points, and it intersects the flat line representing k_{cat} = 33 s^{-1} at 8.6 μM AADP$^+$.

phosphate). The partially reduced enzyme loaded into the stopped-flow instrument contained 40−50% reduced flavin and accumulated approximately 30% flavin semiquinone during the time required for set up (Figure 2). The kinetics of the oxidative half-reaction using this partially reduced thioredoxin reductase were still triphasic. The rate of the catalytically competent phase (phase 2) was slowed to a value near k_{cat} at the pH of the experiment (third and fourth lines of Table 1) and was essentially independent of the concentration of thioredoxin. While these experiments show that a relatively small change of 0.6 pH unit is sufficient to affect the rate of the oxidative half-reaction, it still cannot account for the rate of ~70 s^{-1} observed in the absence of NADP$^+$.

To determine whether the difference in oxidative half-reaction rates between enzyme fully reduced with X/XO/BV and enzyme partially reduced with 1 equiv of NADPH was due to the redox state of the enzyme, thioredoxin reductase reduced with 1.2 equiv of sodium dithionite was used in the oxidative half-reaction at pH 7.6. This produced an enzyme sample which was partially reduced (30% reduced flavin, 48% oxidized flavin, 22% flavin semiquinone), but which had no pyridine nucleotide present. The resultant kinetics were essentially the same as those observed with fully reduced enzyme (fifth line in Table 1). This shows that the redox state of the enzyme does not affect the oxidative half-reaction significantly. Instead, it is likely that the presence of NADP$^+$ was the cause of the decrease in the observed rate of the second phase in experiments where NADPH was the reductant.

It should be noted that while the use of sodium dithionite to partially reduce thioredoxin reductase led to the formation of semiquinone, this did not affect the kinetics of the reaction as compared to fully reduced enzyme, because semiquinone is neither reduced nor oxidized at rates comparable to k_2. This agrees with an earlier conclusion from this laboratory that semiquinone is not active in catalysis (Zanetti et al., 1968). Hence, it is likely that the semiquinone formed in the earlier experiments when NADPH was used as the reductant did not affect the kinetics from those experiments, either.

To further test the hypothesis that pyridine nucleotide causes a decrease in the observed rate of the oxidative half-reaction, it was necessary to use an NADP$^+$ analog which would have the ability to bind like NADP$^+$ but which would not undergo redox chemistry. In experiments in which NADP$^+$ was added to thioredoxin reductase reduced by sodium dithionite prior to use in the oxidative half-reaction, the rate of the main phase was clearly slowed, but a more detailed interpretation was difficult due to the formation of NADPH. The analog chosen to circumvent this was AADP$^+$, which differs from NADP$^+$ in that it has been substituted with an amino group in the 3 position of the pyridinium ring. Electron donation by the amino group of AADP$^+$ as compared to electron withdrawal by the amide of NADP$^+$ leads to an increase in electron density in the pyridinium ring, causing the 4 position of the ring to be less electrophilic (Anderson et al., 1959; Anderson & Kaplan, 1959).

Thioredoxin reductase was fully reduced with X/XO/BV and then mixed with increasing amounts of AADP$^+$ at pH 7.6 prior to use in the oxidative half-reaction. Only one concentration of thioredoxin was used in this experiment (205 μM after mixing). The oxidation of flavin once again contained three phases similar to those seen in previous experiments. The observed rate for the second phase decreased with increasing concentration of AADP$^+$ (Figure 3). A linear regression of the first six points intersects a line drawn at k_{cat} = 33 s^{-1} at 8.6 μM AADP$^+$, which is nearly 1 equiv (1 equiv = 10.3 μM). The rate at 1 equiv of AADP$^+$ was 34.7 ± 6.1 s^{-1} and is essentially equal to k_{cat} (33 s^{-1}). We believe that this curve indicates essentially stoichiometric binding of AADP$^+$ to reduced thioredoxin reductase, with a K_d of ≤1 μM. Thus, the maximal effect on k_2 is reached by about 1 equiv of AADP$^+$.

To aid in determining how well the oxidative half-reaction results obtained in the presence of AADP$^+$ could be compared to those obtained in the presence of NADP$^+$, an attempt was made to measure the binding affinities of the two pyridine nucleotides with thioredoxin reductase. As shown in Figure 4, AADP$^+$ is able to bind to oxidized thioredoxin reductase and perturb its flavin spectrum. The

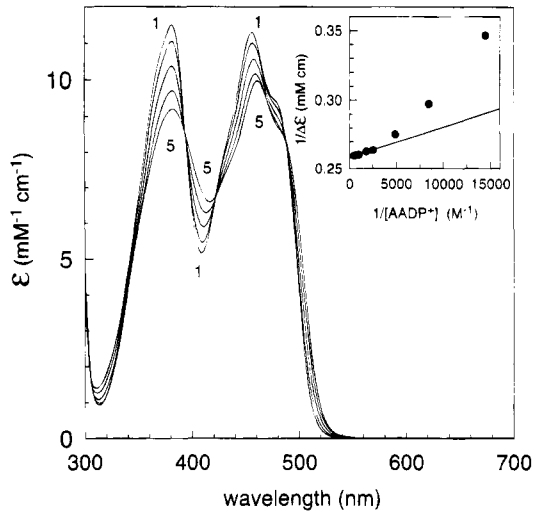

FIGURE 4: Determination of the binding affinity of $AADP^+$ to oxidized thioredoxin reductase. Enzyme was 40 μM in 0.1 M Na/K phosphate, pH 7.6, at 25 °C. Concentrations of $AADP^+$ are (1) 0, (2) 9.9, (3) 29.8, (4) 69.2, (5) 555 μM. More data were collected but have been omitted from this plot for clarity. Inset: A double-reciprocal plot showing the determination of the end point of the titration. The line represents a linear regression of the first four points of the plot, where the concentration of $AADP^+$ was much greater than the concentration of enzyme. This end point was used to calculate a K_d of 11.8 ± 2.0 μM as described in Materials and Methods.

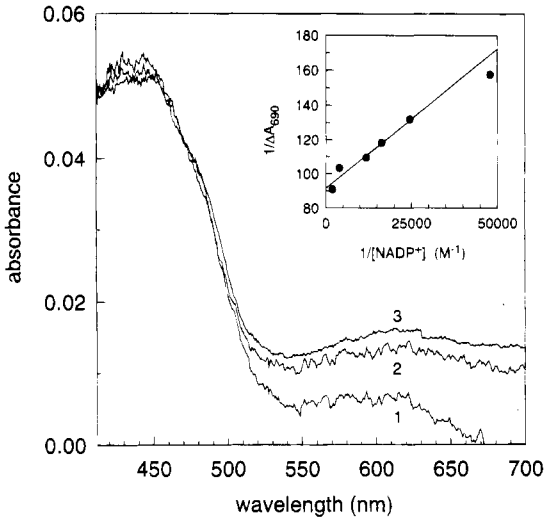

FIGURE 5: Spectra showing the increasing formation of the reduced flavin–$NADP^+$ charge-transfer complex, upon which was based the determination of the K_d for $NADP^+$ to reduced enzyme. Enzyme was reduced by X/XO/BV and sodium dithionite at pH 7.6 prior to rapid mixing with $NADP^+$. Final concentration of enzyme was 10 μM. Actual data used to calculate the K_d came from the dead time absorbances at 690 nm from the single-wavelength data collected on the photomultiplier tube (see inset), which gave better error values. Curve 1, starting enzyme; curve 2, spectrum scanned immediately after the dead time with 20.0 μM $NADP^+$; curve 3, spectrum scanned immediately after the dead time with 40.5 μM $NADP^+$. Inset: A double-reciprocal plot showing the determination of the end point of the titration. The line represents a linear regression of the first four data points in the plot, where the concentration of $NADP^+$ was much greater than the concentration of enzyme. This end point was used to calculate a K_d of 14.0 ± 2.2 μM as described in Materials and Methods.

dissociation constant for $AADP^+$ with the oxidized enzyme was determined to be 11.8 ± 2.0 μM as described in the Materials and Methods. Unfortunately, the binding affinity of $AADP^+$ with reduced thioredoxin reductase could not be determined (see Materials and Methods). However, as described earlier, the fact that $AADP^+$ at substoichiometric concentrations is able to significantly affect the oxidative half-reaction suggests that the binding of $AADP^+$ to reduced thioredoxin reductase is very tight.

The dissociation constant for $NADP^+$ with oxidized thioredoxin reductase was determined to be 87.1 ± 2.5 μM, using the same methods as for $AADP^+$. The dissociation constant for $NADP^+$ with reduced thioredoxin reductase had to be estimated by rapid reaction techniques. The formation of the reduced flavin–$NADP^+$ charge-transfer band at 690 nm gives a direct measure of the extent of binding of $NADP^+$ (Figure 5). From these data, a value for the K_d of 14.0 ± 2.2 μM was obtained. While this result does not indicate that $NADP^+$ binds stoichiometrically to reduced thioredoxin reductase, it is still evident that $NADP^+$ binds more tightly to the reduced than to the oxidized enzyme.

DISCUSSION

Partially reduced thioredoxin reductase is a mixture of four species: E_{ox}, $E(S)_2FADH_2$, $E(SH)_2FAD$ and $E(SH)_2FADH_2$ with the two two-electron-reduced species predominating in enzyme reduced with 1 equiv of NADPH (O'Donnell & Williams, 1983). Only the latter two forms are capable of dithiol/disulfide interchange with thioredoxin. Intramolecular electron transfer must precede interchange in $E(S)_2FADH_2$. Thus, the oxidative half-reaction between reduced thioredoxin reductase and oxidized thioredoxin is composed of two stages, reduced flavin reoxidation and interchange. The rates reported in this paper, as well as in the earlier study (Navarro et al., 1991), are observations of the reoxidation of reduced flavin. The work reported here shows that the level of reduction of thioredoxin reductase does not influence the observed rates. These results suggest that the redox state of the flavin does not affect the dithiol/disulfide interchange between oxidized thioredoxin and the dithiol species of enzyme, and that the same reactive species of enzyme are present at intermediate reduction states as are present at full reduction.

The three-dimensional structure of oxidized thioredoxin reductase has been determined (Waksman et al., 1994). It presents several puzzles particularly with respect to the work reported here and earlier (Navarro et al., 1991) where flavin reoxidation is observed following dithiol/disulfide interchange between reduced thioredoxin reductase and oxidized thioredoxin. Whereas, in the related enzymes glutathione reductase (Karplus & Schulz, 1987) and lipoamide dehydrogenase (Mattevi et al., 1991), the structures reveal a clear path of electrons from reduced pyridine nucleotide to the substrate disulfide via the isoalloxazine ring and the redox active disulfide, this is not the case with thioredoxin reductase. The two thiols in thioredoxin reductase that form the active center disulfide are in the pyridine nucleotide domain rather than in the FAD domain as they are in the other members of this enzyme family (Russel & Model, 1988). Moreover, they are interposed between the pyridine nucleotide binding site and the *re* side of the flavin ring system and are not accessible by thioredoxin. Thus, a sizable conformational change appears to be required during catalysis to allow the pyridinium ring to approach the isoalloxazine ring and to allow dithiol/disulfide interchange with thioredoxin to occur. The structure indicates that this conforma-

tional change can be accomplished by the unimpeded rotation of the pyridine nucleotide domain 66° with respect to the FAD domain, resulting in the juxtaposition of the domains as they are in glutathione reductase (Waksman et al., 1994). Such a rotation moves the dithiol out to the solvent for interchange with the protein substrate, thioredoxin, and moves the nicotinamide ring of the bound NADPH adjacent to the isoalloxazine ring for efficient electron transfer. By contrast, in the conformation actually observed in the crystal, the FAD and the redox-active disulfide are juxtaposed for efficient electron transfer (Waksman et al., 1994). This means that the conformational change must be reversed following interchange in order for the newly formed enzyme disulfide to oxidize the flavin, giving the visible signal which is observed in these experiments. While conformational changes can be fast (Gerstein et al., 1994), no direct evidence exists to show whether this is the case in thioredoxin reductase. Thus, the rate of oxidation of flavin may be limited either by dithiol/disulfide interchange or by the conformational change which alternately allows the enzyme active site sulfurs to be oxidized by thioredoxin and then reduced by the flavin.

We have observed a significant difference in the rates of the main phase of the oxidative half-reaction between reduced thioredoxin reductase and thioredoxin in the presence and absence of pyridine nucleotide. When 1 equiv of NADPH is used as the reductant at pH 7.0 or 7.6, the observed rate of the catalytically competent phase of oxidation is decreased, essentially to k_{cat} at that pH. Since we have shown that the partial reduction state of the enzyme is not the cause of this decrease, it is logically the presence of the pyridine nucleotide. Through the use of the nonreducible analog $AADP^+$, we have shown that this decrease in observed rate of oxidation is linked to the concentration of $AADP^+$ present. This suggests that the complexation of pyridine nucleotides with reduced thioredoxin reductase is able to effect a change in the rate-limiting steps of the oxidation of flavin by thioredoxin. This is the case even when substoichiometric quantities of $AADP^+$ are present, which predicts that the binding to reduced enzyme is very tight. While the K_d for $NADP^+$ to reduced thioredoxin reductase of 14 μM does not indicate stoichiometric binding, it is still clear from other experiments that the presence of 1 equiv of $NADP^+$ is sufficient to cause the observed rate for the second phase of oxidation to decrease to k_{cat}.

We have determined that the slowest competent rate in the reductive half-reaction is 54 s^{-1} under the same conditions as the oxidative half-reaction reported here (Williams et al., 1991). Since k_{cat} under these conditions is 33 s^{-1}, it seems clear that no step in either half-reaction is solely rate limiting. The questions to be answered, then, are why the presence of pyridine nucleotide causes the decrease in the observed rate of oxidation and whether these results are in keeping with the idea that this enzyme proceeds by a ping-pong mechanism in steady-state catalysis.

The presence of pyridine nucleotide could affect the oxidative half-reaction in one of two ways. First, it could form a complex with thioredoxin reductase which must break down before the oxidative half-reaction can proceed. This would mean that turnover probably occurs via a ping-pong mechanism as shown in eq 1, where the first species shown is the starting species in the oxidative half-reaction in the presence of pyridine nucleotide. In this mechanism, if the release of the oxidized pyridine nucleotide occurs at a rate comparable to the rate-limiting step(s) of the oxidative half-reaction, the observed rate of oxidation of flavin would be a combination of the two slowest steps in the overall reaction (marked by an * in eq 1). If the release of pyridine nucleotide were the slowest phase of the reductive half-reaction (see above), this would allow the calculation of an observed rate of $(54 \times 70)/(54 + 70) = 30$ s^{-1} for a classic ping-pong mechanism (Palmer & Massey, 1968).

$$E_{red}{-}NADP^+ \overset{NADP^+}{\underset{*}{\rightleftharpoons}} E_{red} + Tr_{ox} \rightleftharpoons E_{red}{-}Tr_{ox} \underset{*}{\rightleftharpoons} E_{ox} + Tr_{red} \quad (1)$$

Two lines of evidence indicate that the mechanism set forth in eq 1 does not apply to thioredoxin reductase. First, in the absence of pyridine nucleotide, the rate of the oxidative half-reaction is about 70 s^{-1}. Pyridine nucleotide binding, in what appears to be a stoichiometric fashion, slows the rate to a value of approximately 35 s^{-1} (Figure 3). The fact that this value is not zero indicates that dithiol/disulfide interchange can take place with pyridine nucleotide bound. Second, further study of the reductive half-reaction of thioredoxin reductase (to be reported separately) shows that during the slowest phase ($k_{obs} = 54$ s^{-1}) there is formation of a long-wavelength band characteristic of a $FADH_2$–$NADP^+$ charge-transfer complex. This indicates that this step is not simply the release of $NADP^+$.

The second possibility is that pyridine nucleotide could form a complex with thioredoxin reductase where the complex would be the reactive species in the oxidative half-reaction. In this case, through some unknown mechanism pyridine nucleotide bound to the enzyme could cause the rate-limiting step of the oxidative half-reaction to be slowed to k_{cat}. This would predict an ordered mechanism which proceeds through a ternary complex as shown in eq 2, where the slowest step is marked by an * and is equal to k_{cat}. Such a mechanism can give parallel lines in Lineweaver–Burk plots given certain assumptions, and so this mechanism is not necessarily at odds with the steady-state kinetics (Palmer & Massey, 1968). The validity of these assumptions will require a more complete picture of the enzyme reaction mechanism. It should also be noted that this mechanism predicts that the observed value of enzyme oxidation would not go to zero in the presence of pyridine nucleotide, since the reduced enzyme–$NADP^+$ complex is competent to participate in the oxidative half-reaction.

$$E_{red}{-}NADP^+ + Tr_{ox} \rightleftharpoons E_{red} - NADP^+ - Tr_{ox} \underset{*}{\rightleftharpoons} E_{ox} + \text{products} \quad (2)$$

It is also interesting to note that the binding of $NADP^+$ to the reduced enzyme is significantly tighter than to the oxidized enzyme. It has been shown for fully reduced glutathione reductase and lipoamide dehydrogenase that the flavin actually exists in an anionic form (van den Berg et al., 1984). If this is also the case for thioredoxin reductase, the negative charge may cause tighter binding of the positively charged $NADP^+$ relative to oxidized enzyme. The tighter binding of $NADP^+$ to reduced thioredoxin reductase may favor the ternary complex mechanism for thioredoxin reductase. NADPH could reduce the enzyme, leaving a

reduced enzyme−$NADP^+$ complex. With its tighter binding, $NADP^+$ could stay bound until thioredoxin oxidized the flavin, at which time the binding of $NADP^+$ would be weakened, allowing $NADP^+$ to be released in preparation for the binding of the next NADPH. Actually, the differential binding of $NADP^+$ to oxidized and reduced enzyme need not be invoked to support eq 2. Since $AADP^+$ is not able to participate in redox chemistry, it is clear that its effect on the oxidative half-reaction is produced solely by binding to the enzyme. Thus, even if NADPH were to bind to reduced thioredoxin reductase before thioredoxin is able to oxidize the enzyme, it should have the same effect on catalysis as do $AADP^+$ and $NADP^+$, provided the positive charge on the oxidized pyridine nucleotides is not required for their effect on the oxidative half-reaction.

It has been shown that the rate-limiting step in catalysis for thioredoxin reductase does not lie entirely in the isolated oxidative half-reaction (i.e., in the absence of pyridine nucleotide). It has also been shown that even equimolar concentrations of $NADP^+$ can significantly affect the rate-limiting step(s) in the oxidative half-reaction. While it is not yet possible to determine the complete mechanism of thioredoxin reductase, the profound influence of pyridine nucleotides suggests that turnover may proceed via an ordered mechanism which passes through a ternary complex.

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