Mixed Disulfide Intermediates during the Reduction of Disulfides by *Escherichia coli* Thioredoxin

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ABSTRACT: The reduction of disulfides by thioredoxin involves a two-step mechanism. The first step features an intermolecular attack of Cys32 of thioredoxin on the disulfide with formation of a protein mixed disulfide and release of 1 equiv of thiol. The second step involves intramolecular breakdown of the mixed disulfide intermediate via attack of Cys35 with concomitant formation of the oxidized protein and release of a second equivalent of thiol. Study of mixed disulfide intermediates for *Escherichia coli* thioredoxin is exceedingly difficult because the second step is highly favorable. We have studied these intermediates via two approaches. First, Cys35 can be mutated to the similar but chemically nonreactive residue serine. This precludes breakdown of the intermediate. Second, "mass action trapping" techniques can be used because the second step of the mechanism is first-order in the forward direction and second-order in the reverse direction. This has yielded a thermodynamic breakdown of the reaction into its two component steps. Results for reaction of thioredoxin and 2-hydroxyethyl disulfide indicate that about half of the free energy change for the entire process is associated with the first step. Comparison with a small molecule cysteine analog suggests that significant interactions stabilize the mixed disulfide intermediate. Two-dimensional NMR analysis of the C35S thioredoxin 32C−β-mercaptoethanol mixed disulfide shows packing interactions between the mixed disulfide moiety and Trp31 and Ile75. Additionally, studies with C35S thioredoxin show that substitution of the cysteine residue slightly perturbs the equilibrium for the first step in the reaction. Analysis using P34S thioredoxin, a mutant with a drastically higher reduction potential, indicates that most, although not all, of the change is affected in the second step of the reaction. This type of analysis should be useful in structure−function studies for thioredoxin and other oxidoreductases in the thioredoxin superfamily.

Escherichia coli thioredoxin is a 108 residue protein that contains a single active site disulfide bond that can exist in either the oxidized or the reduced form; its oxidation state is coupled to the NADPH/NADP levels via thioredoxin reductase. The protein in the reduced state can provide reducing equivalents for a plethora of biological reactions including the reduction of protein disulfide bonds [for a review, see Holmgren (1989)]. It is the most studied protein of a large family of oxidoreductases, often called the thioredoxin superfamily, which has recently come under intense scrutiny because it is thought that certain members play a crucial role in disulfide-dependent protein folding *in vivo* (Noiva & Lennarz, 1992; Bardwell & Beckwith, 1993). Protein disulfide isomerase (PDI), DsbA (the *E. coli* disulfide isomerase), and glutaredoxin are all well-studied members of this family. These proteins may aid in folding by catalyzing the formation of native disulfide bonds and additionally by catalyzing the isomerization of kinetically trapped folding intermediates that may occur with proteins that contain multiple disulfide bonds (Weissman & Kim, 1993).

The reduction potentials for the members of this superfamily vary widely. Thioredoxins have the lowest reduction potentials (−0.23 to −0.26 V; Holmgren, 1968; Berglund & Sjoberg, 1970; Gleason, 1992) while DsbA and PDI have considerably higher values (−0.089 and −0.11 V, respectively; Hawkins et al., 1991; Wunderlich & Glockshuber, 1993). While the reduction potential for thioredoxin is close to that of the common dithiol reducing reagent dithiothreitol, the values for DsbA and PDI are much higher than that for β-mercaptoethanol (BME),[1] a reducing agent that must be used in vast excess to reduce protein disulfides (Szajewski & Whitesides, 1980). Consequently, DsbA and PDI have been referred to as oxidants while Trx is usually thought of as a reductant. The large differences in activity are even more surprising in light of the available structural data. The structures of the oxidized forms for Trx and DsbA have been solved by X-ray crystallography (Katti et al., 1990; Martin et al., 1993), and the 14 atoms of the active site disulfide ring superimpose with a rms deviation of 0.1 Å. The

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[1] Abbreviations: Trx, thioredoxin; BME, β-mercaptoethanol; BMEDS, 2-hydroxyethyl disulfide; P_{mds}, protein mixed disulfide intermediate; P_{ox}, protein with disulfide bond intact; P_{red}, protein in the reduced state; Gdn-HCl, guanidine hydrochloride; NMR, nuclear magnetic resonance; ACM, *N*-acetylcysteine methylamide; DQF-COSY, double-quantum-filtered correlated spectroscopy; NOESY, nuclear Overhauser spectroscopy; NOE, nuclear Overhauser effect; TPPI, time-proportional phase incrementation.

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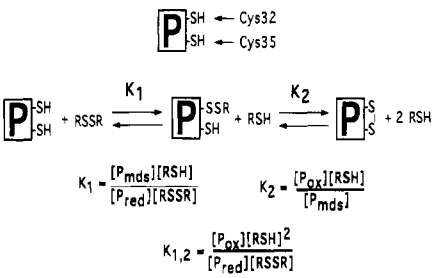

FIGURE 1: Reaction mechanism for the reduction of a disulfide by thioredoxin.

structure of the reduced form of Trx has also been solved by NMR methods and is very similar to that of the oxidized form (Dyson et al., 1990).

The mechanism for reduction of a disulfide by thioredoxin is shown in Figure 1. The first step features attack of Cys32 on the disulfide to be reduced with formation of a protein mixed disulfide intermediate and release of 1 equiv of thiol. Cys32 was shown to be the exclusive attacking group by chemical reactivity studies and by its abnormal pK_a of 6.7 (Kallis & Holmgren, 1980). Structural studies later supported this since in both oxidized and reduced Trx Cys35 is not accessible to solvent while Cys32 is (Katti et al., 1990; Dyson et al., 1990). Similar relative reactivities for the active site thiols have been observed for DsbA and PDI (Hawkins & Freedman, 1991; Nelson & Creighton, 1994). This first step is second-order in both the forward and reverse directions. Step 2 features attack of the γ-sulfur atom of Cys35 on the mixed disulfide moiety. This results in formation of the oxidized protein and release of a second equivalent of thiol. This step is first-order in the forward direction and second-order in the reverse direction. Since each step is a disulfide exchange reaction, they can be described by individual equilibrium constants. Here we use K_1 and K_2 to describe the first and second steps in the reaction and $K_{1,2}$ to describe the overall process. Note that $K_{1,2} = K_1 \times K_2$. $K_{1,2}$ is related to the difference in reduction potential of the protein and thiol/disulfide pair. If the reduction potential for the thiol/disulfide pair (or other redox partner) is known, then the reduction potential can be determined by measuring the overall equilibrium described by $K_{1,2}$.

Characterization of the redox activity for various members of the thioredoxin superfamily usually involves determination of only the reduction potential. A more complete description would involve not only the reduction potential, which is independent of the substrate molecule, but also evaluation of the equilibrium constants K_1 and K_2 which could potentially furnish information about protein−substrate interactions. The determination of each individual equilibrium constant could also furnish information about whether changes in the reduction potential are due to changes in the oxidized or reduced state or both. For thioredoxin, study of the individual steps is particularly difficult because the second step of the reaction is very favorable. As a result, the mixed disulfide intermediate is not populated significantly at equilibrium under normal conditions. We use two methods to overcome this problem. (1) Mutagenesis at position 35 to the similar but chemically nonreactive serine residue precludes the second step from occurring. Step 1 can then be studied without complication. The second step can be inferred from knowledge of $K_{1,2}$ for the wild-type protein and K_1 of the mutant protein. This approach assumes that mutation at position 35 will not affect the first step equilibrium. We discuss the validity of this assumption and its relevance to activity and structural studies. (2) As mentioned above, the second step of the reaction is first-order in the forward direction and second-order in the reverse direction. Thus, this step can be inhibited by high concentrations of the product thiol. When, [RSH] = K_2, the oxidized protein and the mixed disulfide intermediate will be equally populated. We have used BME and BMEDS as the thiol/disulfide pair. The high solubility of both molecules allows for concentrations appreciable to K_2 to be obtained.

Here we present detailed methods for populating the mixed disulfide intermediate of thioredoxin and a thermodynamic description of both steps in the reduction of BMEDS by thioredoxin. It is shown that about half of the favorable free energy change for the entire process is associated with the first step. Comparison with a small cysteine analogue suggests significant interactions between the mixed disulfide moiety and the protein, a conclusion supported by two-dimensional NMR analysis. Study of C35S thioredoxin shows that small but significant perturbations of the first step equilibrium do occur when position 35 is changed. Analysis of a mutant thioredoxin, P34S Trx, shows that most of the large increase in the reduction potential for this protein is brought about by changes in the second step. Finally, comparison with published data for DsbA (Zapun et al., 1993) indicates that there are differences, in magnitude and sign, in both steps of the reaction when compared to wild-type thioredoxin. The methods and results presented here should be useful in structure−function studies with thioredoxin and other members of the thioredoxin superfamily.

MATERIALS AND METHODS

Chemicals. Sequanal grade Gdn-HCl was purchased from Boehringer Mannheim. BME was purchased from Sigma and distilled under reduced pressure. BMEDS was synthesized by the Cu^{2+}-catalyzed oxidation of BME as described (Hopton et al., 1968). HPLC grade water and acetonitrile were purchased from J. T. Baker, Inc., and HPLC grade trifluoroacetic acid was purchase from Pierce. (2-Hydroxyethyl)methanethiosulfonate was synthesized and purified as previously described (Wynn & Richards, 1993b).

Proteins. Wild-type thioredoxin was overexpressed and purified as previously described (Hellinga et al., 1992). High-copy plasmids carrying genes for C35S and P34S Trx were generously provided by Marjorie Russel and Peter Model of Rockefeller University, and these proteins were prepared as described (Russel & Model, 1986). Additionally, some P34S Trx for initial experiments was provided by David LeMaster (Northwestern University).

Production of C35S Trx−2-Hydroxyethyl Mixed Disulfide. Reduced C35S Trx at a concentration of 1 mg/mL was mixed with a slight excess (1.1-fold) of (2-hydroxyethyl)methanethiosulfonate in 0.05 M Tris, pH 8.0. After 15 min, the mixture was applied to a G-50 column to remove excess reagent. Both the extent of modification measurement and SDS−PAGE analysis were carried out as previously described (Wynn & Richards, 1993a).

Measurement of Disulfide Exchange Equilibrium Constants. The buffer used throughout these experiments was 0.05 M Tris, 0.001 M EDTA, pH 8.0. All solutions were

degassed and purged with argon prior to use. Samples containing protein, BME, and BMEDS were incubated for 3 h at 25 °C. BME and BMEDS were always in at least a 200-fold molar excess over the protein so that the concentrations were effectively fixed throughout an experiment. Disulfide exchange was quenched by adding equal volumes of reaction mixture and 6.0 M Gdn-HCl, 0.5 M sodium phosphate, pH 2.5. The Gdn-HCl denatures the protein and thus decreases the proximity of the two active site thiols while the low pH quenches disulfide exchange which is dependent upon the thiolate anion concentration. After being quenched, the reaction mixtures were applied to a Vydac C18 reverse phase HPLC column (4.6 mm by 25.0 cm) equilibrated with 35% acetonitrile. Elution occurred over 60 min with a gradient to 65% acetonitrile while the effluent was monitored at 229 nm. All HPLC buffers contained 0.01% trifluoroacetic acid. Relative protein concentrations were determined by integrating the appropriate peak areas using the Dynamax software from Rainin Instrument Co., Inc. Equilibrium constants were determined by linear (K_2) or nonlinear (K_1 and $K_{1,2}$) least-squares fitting to the equations:

$$[P_{mds}]/([P_{mds}] + [P_{red}]) = R/(K_1 + R)$$

$$[P_{ox}]/[P_{mds}] = K_2/[RSH]$$

$$[P_{red}]/[P_{ox}] = R'/(K_{1,2} + R')$$

where R and R' equal [BME]/[BMEDS] and [BME]^2/[BMEDS], respectively. K_2 was fit to a linear equation because the concentration of thiol was limited to approximately $K_2/4$. Thus, a nonlinear least-squares treatment is not justified.

Mass Spectroscopy. A reaction mixture was quenched as described above, and proteins were separated from excess thiol and disulfide using Sep-pak C18 cartridges as previously described (Wynn & Richards, 1993b). The protein mixture was analyzed on a VG BioTech/Fisons (Altrincham, U.K.) triple-quadrapole instrument with an electrospray ionization source (Analytica, Branford, CT).

NMR Spectroscopy. NMR spectra were acquired on a Bruker AM-500. Samples were prepared in 100 mM potassium phosphate, pH 8.0, with 10% D_2O at a concentration of 3.5 mM protein. DQF-COSY and NOESY spectra were recorded at 298 K for a period of 40 h total. The water resonance was suppressed through saturation during the 1.1 s relaxation delay and the mixing time. DQF-COSY (Rance et al., 1983) and Hahn-echo NOESY (Kumar et al., 1980; Bodenhausen et al., 1984; Davis, 1989) spectra were recorded in the phase-sensitive mode using the TPPI method (Drobny et al., 1979; Marion & Wuthrich, 1983). Spectral widths were 6250 Hz in both t_2 (4096 points) and t_1 (512 points). Sixty-four transients were collected for each of the t_1 increments in the DQF-COSY. Ninety-six transients were collected for each t_1 in the NOESY spectrum. The NOESY experiment was run with a 125 ms mixing time. Data were processed on a Silicon Graphics IRIS using FELIX (Biosym, Inc.).

For model building, energy minimization was carried out with a NOE term added to the "parallhdg.pro" parameter set of Xplor (Brunger, 1992). A square well potential function with a target distance range of 3.0−6.0 Å was used for the NOE term. The starting structure was obtained from the reduced thioredoxin structure previously determined (Dyson et al., 1990), and only the Cys32-BME disulfide was actually minimized, the remainder of the structure being held rigid. The disulfide moiety was initially added in an extended conformation. Energy minimization using the conjugate gradient method of Powell (1977) was carried out for 500 cycles. We have assumed in this treatment that the assignment of the methylenes in the BME moiety could be made on the basis of the electronegativity of the bonded atoms (see Results and Discussion). Switching the assignments had little effect on the final model structure probably because a square well potential function was used with a large target distance range. Minimizations starting from several different BME conformations did not significantly alter the final model.

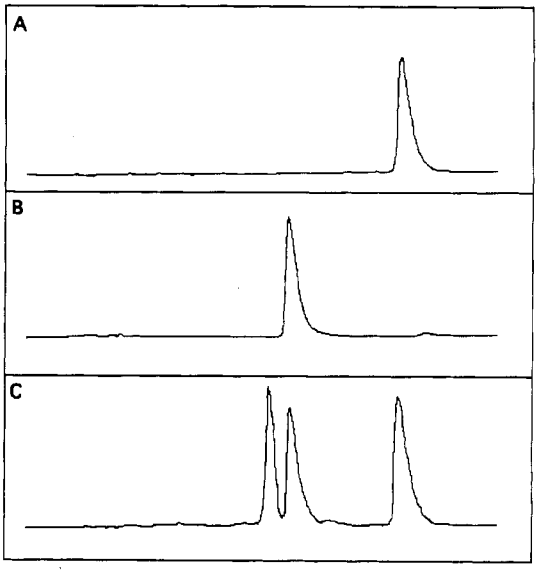

FIGURE 2: C18 HPLC separation of wild-type thioredoxin reaction mixtures. Top panel, oxidized Trx. Middle panel, reduced Trx. Bottom panel, reaction mixture.

RESULTS AND DISCUSSION

Detection of Mixed Disulfide Intermediates. Figure 2 (bottom panel) shows representative chromatograms from the reverse phase HPLC separation of the quenched reaction mixtures for wild-type thioredoxin. Three separate protein peaks are clearly observable under the conditions of our experiments. Two of these line up with the reduced and oxidized forms of the protein (Figure 2, middle and top panels, respectively). When the thiol concentrations are much lower than used here, only peaks that match with the oxidized and reduced protein are observed (data not shown). The peak with the earliest elution time was putatively assigned to be the thioredoxin mixed disulfide intermediate. We discuss below the validity of this assignment.

Mass spectral analysis of the protein component of a reaction mixture as described above (Materials and Methods) indicated only two masses of MW 11 672 ± 5 and 11 746 ± 3 amu. The first is the expected mass of unmodified thioredoxin (reduced and oxidized differ by only 2 amu and thus would not be distinguished). The second mass is higher by 74 amu. This is within experimental error of what would be expected for a BME mixed disulfide to thioredoxin. Thus, the unidentified peak corresponds to a mixed disulfide to only one of the available cysteines in thioredoxin.

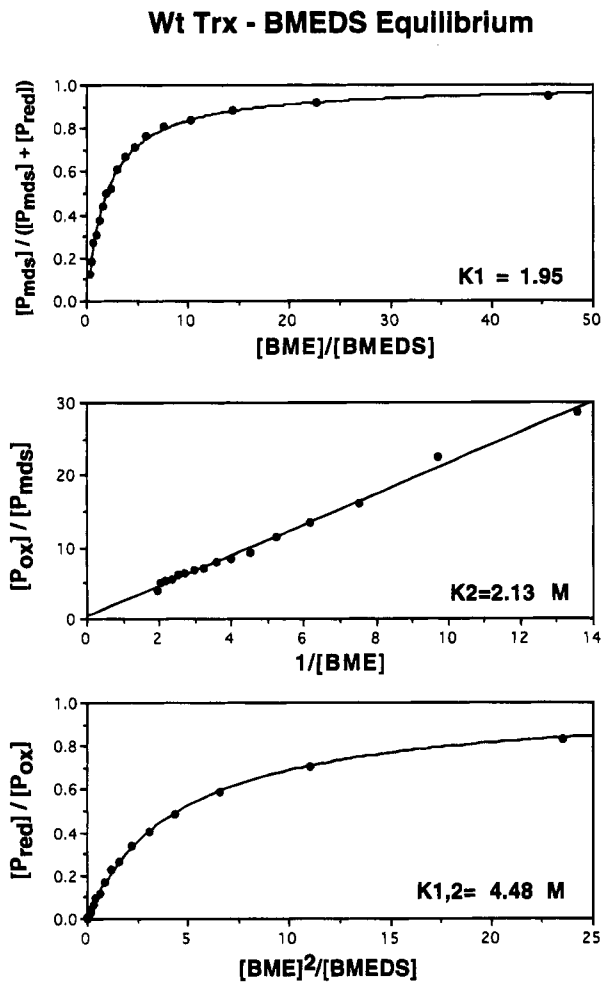

FIGURE 3: Curve fits to experimental data for K_1 (top), K_2 (middle), and $K_{1,2}$ (bottom).

A mixed disulfide to Cys32 as opposed to Cys35 is strongly supported by available physical, chemical, and structural information. The structures of both oxidized and reduced thioredoxin show that Cys35 is buried in both forms of the protein and thus would be inaccessible to thiols and disulfides in the solvent (Katti et al., 1990, Dyson et al., 1990). The fact that Cys35 is buried in both the oxidized and reduced structures suggests that formation of mixed disulfides to this residue is unlikely since deformation of the protein would have to occur to accommodate the new disulfide moiety. Cys32, on the other hand, is partially exposed. Early chemical modification experiments showed Cys32 to be much more reactive and to have an abnormally low pK_a of 6.7 (Kallis & Holmgren, 1980). The low pK_a of cysteine 32 has been confirmed by several techniques including NMR (Dyson et al., 1991), fluorescence spectroscopy (Reutimann et al., 1981), and laser Raman spectroscopy (Li et al., 1993). A Cys32 mixed disulfide structure could remain solvent-exposed or pack against the outside of thioredoxin (as will be discussed below, several hydrophobic residues surround Cys32 and raise the possibility of favorable hydrophobic contacts).

Figure 3 shows curve fits for wild-type thioredoxin while Table 1 lists the derived equilibrium constants. The factors discussed above suggest that formation of a mixed disulfide to Cys35 would be much less favorable than formation of a mixed disulfide to Cys32; i.e., K_1 would be much higher for the Cys32 equilibrium. The good fits for K_1 and K_2 to a model which entails only a single mixed disulfide species, and consequently single equilibrium constants, indicate that no significant amount of mixed disulfide to Cys35 occurs under the conditions in our experiments. The relation $K_1 \times K_2 = K_{1,2}$ serves as an internal check on the validity of the assumed equilibria. For wild-type thioredoxin, $K_1 \times K_2$ is within 8% of $K_{1,2}$.

Table 1: Disulfide Exchange Equilibrium Constants

	WT Trx	P34S Trx	C35S Trx
K_1[a]	1.95	1.02	0.781
K_2[b]	2.13	0.145	
$K_{1,2}$[c]	4.48	0.120	

[a] K_1 is a dimensionless quantity. [b] K_2 is expressed in molar. [c] $K_{1,2}$ is expressed in molar.

Validity of C35S Trx Mixed Disulfides as Models for Reaction Intermediates. Use of the C35S Trx−BMEDS equilibrium as a model for the first step in the wild-type reaction assumes that the mutation at position 35 does not strongly affect the value of K_1. In this study, it is not necessary to rely on this approach in order to determine the individual values of K_1 and K_2 because of the high solubility of BME and BMEDS. However, many potential thiol substrates of thioredoxin, whether protein or nonprotein, will not have the required solubility ($\approx K_2$) to significantly populate the mixed disulfide intermediate. Additionally, structural investigation of the intermediate will be hampered by high concentrations of the thiol and disulfide species as well as by population of the oxidized and reduced forms of the protein. Thus, use of position 35 mutants is likely to be the most useful method of characterizing the mixed disulfide intermediate. Structural studies using cysteine mutants have already been carried out for glutaredoxin (Bushweller et al., 1992; Bushweller et al., 1994) and human thioredoxin (Qin et al., 1995).

Table 1 shows that the K_1 for wild-type thioredoxin and the K_1 for C35S thioredoxin differ by a factor of 2.5. This corresponds to a factor of approximately 0.5 kcal/mol. The difference is not large but significant and warrants caution in interpreting data involved with position 35 mutants. Differential scanning calorimetry studies of reduced thioredoxin and the C32S, C35S mutant show that the melting temperature and free energy of unfolding are similar for the two forms but the enthalpy and entropy are partitioned differently (Ladbury et al., 1994). This behavior may reflect the stronger hydrogen bonding potential of hydroxyls relative to thiols and/or the ionization of Cys32 (p$K_a \approx 7.0$) which presumably would not take place with a serine. Additionally, the high temperatures for the calorimetric experiments may exacerbate the difference between the double serine and reduced form of the proteins. Dyson et al. (1994) have concluded on the basis of NMR analysis that C32S, C35S is similar to the reduced form of the protein although they do note a change in the pK_a of Asp26 (Dyson et al., 1994). It is possible that other changes at position 35, alanine being a good candidate, will perturb the equilibrium less. However, the similarity in the average structures of reduced and C32S, C35S thioredoxin suggests that it is reasonable to use a position 35 mutant as a model for the mixed disulfide intermediate.

Equilibrium Constants for Wild-Type Thioredoxin. The value of K_1 determined for wild-type thioredoxin is 1.95

(Table 1). This represents a single disulfide exchange step between Cys32 and BMEDS. The value of K_2 is 2.13, and this represents the formation of the intramolecular disulfide between the two active site cysteines. The similarity of the two values shows that each step is nearly equally favorable. Thus, the high reducing ability of thioredoxin is due to a high tendency for Cys32 to form mixed disulfides as well as a highly favorable formation of the oxidized protein; i.e., the low reduction potential is due to features of both the oxidized and reduced forms of the protein.

The value determined for K_1 is close to the value to the value determined for *N*-acetylcysteine methylamide (ACM), an analog of cysteine in a peptide chain, and BMEDS under identical conditions (2.05) (Wynn & Richards, 1993b). One might assume this indicates that Cys32 merely sits on the surface of the active site and that the protein provides an unutilized scaffold. However, it has been noted above that Cys32 has an abnormally low pK_a of approximately 7. While this should speed the kinetics of the nucleophilic attack by Cys32, it should disfavor formation of a mixed disulfide intermediate if the pK_a of the released thiol is much higher as is the case with BME:

$$K_{1,\text{apparent}} = [\text{PSSR}][\text{RSH} + \text{RS}^-]/[\text{PSH} + \text{PS}^-][\text{RSSR}]$$

$$K_{1,\text{apparent}} = K_1^\circ[1 + K_{a,\text{RSH}}/[\text{H}^+]]/[1 + K_{a,\text{PSH}}/[\text{H}^+]]$$

where K_1° is the equilibrium constant at low pH and $K_{a,\text{RSH}}$ and $K_{a,\text{PSH}}$ are the ionization equilibrium constants for BME and Cys32, respectively. Since the pK_a's of both BME and ACM are much higher than the pH of these experiments, K_1° is essentially equal to the value quoted above for this equilibrium. Using a pK_a of 7 for Cys32 in reduced thioredoxin and a pK_a of 9.6 for BME (Wilson et al., 1977), one would expect, assuming no other effects from the protein, the value of K_1 to be approximately 10-fold lower than the measured value at pH 8.0 ($\approx$0.2). The protein must provide additional interactions which counterbalance the unfavorable effect of the Cys32 ionization on the first-step equilibrium. It has previously been noted that the thioredoxin active site is surrounded by hydrophobic residues (Katti et al., 1990). Trp31, Pro34, Ile75, and Pro76 are all on the surface and in the vicinity of Cys32. The mixed disulfide intermediate could potentially be stabilized by packing of the hydrocarbon/disulfide moiety against some of these nonpolar residues. Below we describe two-dimensional NMR experiments which support this suggestion. It should be noted that this hydrophobic surface also plays a significant role in the interaction of thioredoxin with its many biological partners (Eklund et al., 1991). The hydrophobic surface may also be important in its ability to refold disulfide-containing proteins.

The value of K_2 is 2.13 M. We hesitate to use the term "effective concentration" for this step or the reaction described by $K_{1,2}$. Effective concentration is a term that is useful in describing entropic effects when comparing intramolecular and intermolecular reactions. Enthalpic effects, which are likely in the context of a protein, would cause the interpretation of these equilibrium constants in terms of effective concentrations to be misleading. Burns and Whitesides (1990) have also pointed out that the enthalpy of a disulfide bond strongly depends on the CSSC dihedral angle and this is also likely to lead to enthalpic effects. Still, the high value of K_2 suggests that the oxidized protein is low in free energy, a fact supported by the relative stabilities of the oxidized and reduced wild-type proteins; oxidized thioredoxin has a T_m that is 12 °C higher than reduced thioredoxin (Holmgren, 1972), and the oxidized form is also more stable to guanidine hydrochloride denaturation (Kelley et al., 1987).

Table 2: Chemical Shift Assignments for the C35S Trx−BME Mixed Disulfide

Trp28	NH 8.46; CαH 4.99; CδH 7.13; Nε1H 10.77; Cζ2H 7.70; CηH 7.29; Cζ3H 6.95; Cε3H 7.15
Ala29	NH 7.01; CαH 3.51; CβH 0.33
Glu30	NH 9.09; CαH 4.10; CβH 2.04
Trp31	NH 6.58; CαH 4.56; CβH 3.18,3.72; CδH 7.45; Nε1H 11.97; Cζ2H 7.39; CηH 7.19; Cζ3H 7.13; Cε3H 7.43
Cys32	NH 6.83; CαH 5.06; CβH 2.70
Ile75	CγH2 1.04,1.56; CγH3 0.87; CδH 0.43
BME	CαH 3.26,3.35; CβH 3.99,4.11

NMR of the C35S Trx−BME Mixed Disulfide. Chemical shift assignments were aided by the known structures of oxidized (Katti et al., 1990) and reduced (Dyson et al., 1990) thioredoxin and by the assignments for the protein in each form (LeMaster & Richards, 1988, Dyson et al., 1989) as well as the partial assignment of C32S, C35S thioredoxin (Dyson et al., 1994). We have made assignments for residues 28−32, 75, and the BME moiety. The chemical shifts for these residues are listed in Table 2. Trp28 and Trp31 were identified from the respective Nε protons which resonate far downfield. These were distinguished by NOEs to Ala29 and similarity to the published chemical shifts for the oxidized and reduced protein. Ala29, Glu30, Cys32, and Ile75 could be assigned on the basis of coupling and NOE patterns. The $C\beta H_3$ of Ala29 was initially assigned on the basis of NOE intensity to the Trp31 side chain and was confirmed by the expected coupling pattern. The NH of Glu30 was identified by NOE intensity to the NH of residue 31 and the CαH of residue 29. The Glu30 CαH showed the expected NOE cross-peaks to the Trp28 Cζ3H and to the Trp31 NH. The CβH for residue 30 was assigned based on coupling to CαH and NOE information to its own NH. The NH of Cys32 showed the expected NOE intensity to Trp31 NH and Ala29 CβH3. The Cys32 CβH showed NOEs to Ala29 NH and $C\beta H_3$ and also to its own NH. The CδH3 of Ile75 was assigned from NOE cross-peaks to the $C\beta H_3$ and NH of Ala29. The remainder of the side chain was assigned with coupling information. The BME moiety was assigned on the basis of (1) the expected coupling in an A2B2 spin system (no coupling in the Cα or aliphatic region could be unambiguously assigned), (2) the approximate similarity in chemical shift relative to the *N*-acetylcysteine methylamide−BME mixed disulfide and (2-hydroxyethyl)methanethiosulfonate (Wynn & Richards, 1993b), (3) NOE peaks to nearby active site residues Trp31 (Cζ3H, CηH, Cζ2H, and Cε3H) and Ile75 ($C\delta H_3$ and $C\gamma H_3$). We could not assign unambiguously the protons on the BME moiety but on the basis of electronegativity suggest that the 3.99 and 4.11 ppm resonances are for the methylene group adjacent to the hydroxyl group (CβH) while the 3.26 and 3.35 ppm resonances are for the sulfur-bonded methylene (CαH). The two protons in each methylene group are resolved, and this suggests that this group is undergoing little motion.

Comparison of the chemical shift values for the BME mixed disulfide protein with the assigned values for reduced

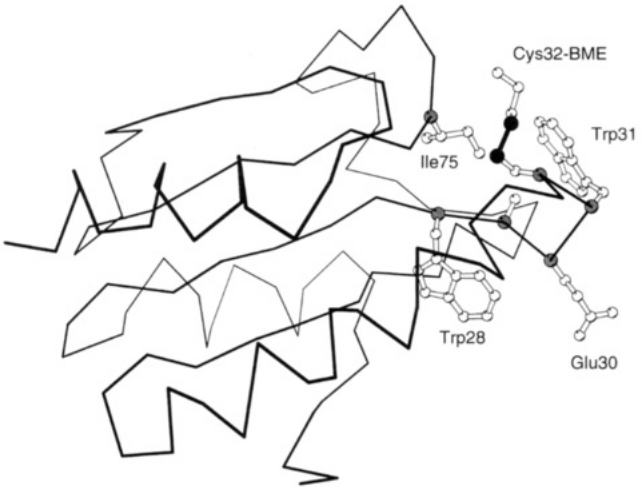

FIGURE 4: α carbon trace of the model for C35S Trx−BME mixed disulfide. The side chains of Trp28, Ala29, Glu30, Trp31, Cys-BME32, and Ile75 are shown in white. α carbons are gray, and sulfur atoms are black.

and oxidized thioredoxin (Dyson et al., 1989) indicates that this region of the protein does not undergo any large conformational change upon mixed disulfide formation. The largest change in chemical shift, not counting Cys32 which undergoes a covalent bond change, is for the Nϵ1 proton of Trp31 ($\approx$0.5 ppm) and probably reflects a change in the hydrogen bond strength between this group and the side chain of Asp61. Since the chemical shifts for the rest of this side chain are largely similar to the other forms of thioredoxin, the overall position of residue 31 is most likely unperturbed. The only other changes greater than 0.3 ppm are the CαH of Trp28 (when compared to the reduced form) and the NH of Ala29 (compared to the oxidized form). Figure 4 shows an α carbon trace of thioredoxin with the assigned residues included. In general, the differences in chemical shift between the mixed disulfide form and either the oxidized or the reduced form are comparable to the difference between the oxidized and reduced forms. Thus, all three forms of the protein have highly similar structures.

We have detected NOE intensity between the BME moiety and residues Trp31 and Ile75. All four protons of the BME group show intensity to the CδH$_3$ of Ile75. Dipolar contacts between Trp31 and the BME protons include the following: BME resonance at 3.26 ppm to Trp31 Cζ3H, CηH; BME resonance at 3.35 ppm to Trp31 Cζ3H, CηH, Cζ2H, Cϵ3H; BME resonance at 3.99 ppm to Trp31 CηH. Energy minimization (as described under Materials and Methods) has shown that contact between the BME moiety and the side chains of Trp31 and Ile75 can occur without much rearrangement (see Figure 4). It is likely that similar interactions will occur between residues 31 and 75 in other thioredoxin mixed disulfides whether they are protein or nonprotein in nature.

Comparison of Wild-Type and P34S Thioredoxin. The K_1, K_2, and $K_{1,2}$ values for P34S thioredoxin are listed in Table 1. There are changes in both steps of the reaction relative to the wild-type protein although the change in step 2 (14.7-fold) is much larger than that for step 1 (1.91-fold). One interpretation of these data would assume that the interactions in the P34S mixed disulfide intermediate are slightly less stabilizing than those in the wild type and the interactions in the oxidized form of P34S are considerably less stabilizing than the wild type. However, the observed equilibrium constants could also be accounted for by postulating changes in any two or all three forms.

The disulfide exchange equilibrium between the *E. coli* periplasmic protein DsbA and glutathione has been studied by Zapun et al. (1993). The similarity between the reduction potentials and pK_a's of glutathione and BME suggests that comparison with the two is reasonable (Lees & Whitesides, 1993). The authors find that both steps in the reaction are much less favorable than we observe here for wild-type thioredoxin ($K_1 = 3.7 \times 10^{-3}$ M and $K_2 = 1.9 \times 10^{-2}$ M). On the basis of the differences in stabilities between the oxidized and reduced forms of DsbA (the reduced form is 5.4 kcal/mol more stable than the oxidized form), Wunderlich et al. (1993) have suggested that the oxidizing properties (high redox potential) of DsbA result mainly from a tense disulfide bond in the oxidized species. One would expect only a change in K_2 if this were the case instead of the large differences in K_1 and K_2 that we observe. Like the case with P34S thioredoxin, it is possible that the relatively high reduction potential of DsbA is also a manifestation of stabilizing interactions in the reduced state. Put another way, the gain in stability after reduction of DsbA is just as likely to result from the addition of stabilizing interactions as the removal of unfavorable ones. The fact that K_1 is low raises this alternate possibility.

Conclusion. We have found a method which we entitle "mass action trapping" to populate the mixed disulfide intermediate of thioredoxin and BME even though breakdown of this intermediate is very favorable. It is likely that this intermediate is stabilized by hydrophobic interactions between the disulfide moiety and Trp31 and Ile75 of the protein. Additionally, we find that mutation of Cys35 does significantly but slightly affect the disulfide exchange properties of Cys32. Thus, single serine mutants may be used as models for mixed disulfide intermediates, but results should be interpreted with caution. Other amino acids at position 35 may prove less perturbing in the future. Analysis of P34S thioredoxin shows that the higher reduction potential of this protein is due to changes in both the first and seconds steps although the majority of change is manifested in the second step. Finally, comparison of wild-type thioredoxin with DsbA shows large unfavorable changes in each step of the reaction. Thus, the different reactivity of DsbA results from a lower tendency to form mixed disulfides as well as less favorable intramolecular disulfide bond formation.

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