On the Reactivity and Ionization of the Active Site Cysteine Residues of *Escherichia coli* Thioredoxin†

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ABSTRACT: Within various proteins of the thioredoxin family, the stability of the disulfide bond formed reversibly between the two active site cysteine residues, one accessible and one buried, varies widely and is directly correlated with the pK_a value of the accessible cysteine thiol group. If applicable to thioredoxin, its stable disulfide bond would imply that its accessible thiol group should have a high pK_a value, whereas it has long been considered to be about 6.7, largely on the basis of the pH dependence of its reactivity. Such kinetic data are shown to be inconsistent with known pK_a values in this case; the rate constants may reflect effects in the transition state for the reaction, which is catalyzed by thioredoxin, rather than the protein itself. Ionization of the thioredoxin thiol groups was measured indirectly by the pH dependence of the equilibrium constant for their reaction with glutathione and directly by detection of the thiolate anion by its UV absorbance. Both observations indicated that both cysteine thiol groups of thioredoxin ionize with apparent pK_a values in the region of 9−10 and that their ionization is not linked strongly to that of any other groups. This conclusion is not incompatible with the other data available and would make thioredoxin consistent with the relationship between thiol group ionization and disulfide stability observed in other members of the thioredoxin family.

The thioredoxin fold, named after the protein in which it was first observed, has been adapted for use in a number of proteins that act on substrate thiol and disulfide groups (Martin, 1995): thioredoxin (Holmgren et al., 1975; Katti et al., 1990); glutaredoxin (Xia et al., 1992); the known catalysts of protein disulfide formation, protein disulfide isomerase (PDI) (Edman et al., 1985; Kemmink et al., 1995), DsbA (Martin et al., 1993), and probably DsbC (Zapun et al., 1995; Frishman, 1996). These proteins function using two cysteine residues in sequences of the type -Cys-X-Y-Cys- to cycle between the dithiol and disulfide forms, designated here for thioredoxin as Trx_{SH}^{SH} and Trx_{S}^{S}, respectively.[1] Only the first of these sulfur atoms is accessible in the thioredoxin motif, and only it reacts readily by thiol−disulfide exchange with other thiol and disulfide groups; the second sulfur atom is completely buried and generally reacts only with the first, to form the intramolecular disulfide bond between them (Kallis & Holmgren, 1980).

The proteins of the thioredoxin family differ markedly in the stabilities of the disulfide bonds that are formed between the two cysteine residues at their active sites. For example, thioredoxin functions as a reductant and has a relatively stable disulfide bond (Holmgren, 1981), whereas DsbA acts as an oxidant and has a disulfide bond that is 7 kcal/mol less stable (Zapun et al., 1993; Wunderlich & Glockshuber, 1993). Yet the two disulfide bonds are indistinguishable structurally (Martin et al., 1993). A major goal is to understand the physical basis of the differences in stabilities, reactivities, and functions of the homologous disulfide bonds of the members of the thioredoxin family.

The various thioredoxin-like proteins differ in the residues X and Y in the sequences -Cys-X-Y-Cys-: thioredoxin, -Gly-Pro-; glutaredoxin, -Pro-Tyr-; PDI, -Gly-His-; DsbA, -Pro-His-; and DsbC, -Gly-Tyr-. Changing these two residues can alter the stability of the disulfide bond dramatically (Gleason, 1992; Lundström et al., 1992; Wunderlich, 1994; Grauschopf et al., 1995), but only in the folded protein (Siedler et al., 1993). For example, putting the thioredoxin sequence -Gly-Pro- into DsbA increases the stability of its disulfide bond by a factor of about 10^3 (Wunderlich, 1994). It might be expected that these effects would be due to varying amounts of conformational strain in the disulfide bond, but this appears not to be the case. The observation that the accessible cysteine thiol group of reduced DsbA has a very low pK_a value of about 3.5, rather than the usual value of about 8.7, suggested that the favorable electrostatic interactions that must be responsible for this greatly decreased pK_a value would stabilize the reduced form of the protein, but not the disulfide form (Nelson & Creighton, 1994). Therefore, the differences in disulfide stability would not be due to differences in the disulfide bonds themselves,

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH and GSSG, reduced and oxidized forms of glutathione, respectively; HPLC, high-pressure liquid chromatography; NTB, 2-nitro-5-thiobenzoic acid; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; Trx_{SH}^{SH}, Trx_{S}^{S}, Trx_{SH}^{SSG}, Trx_{SSG}^{SH}, and Trx_{SSG}^{SSG}, *E. coli* thioredoxin with, respectively, its two active site cysteine thiol groups, a disulfide bond between them, and Cys32, Cys35, or both forming a mixed disulfide bond with glutathione (the upper sulfur atom is that of Cys32 in each case); Trx_{SH}^{OH}, Trx_{OH}^{SH}, and Trx_{OH}^{OH}, respectively the variants of *E. coli* thioredoxin with Cys32, Cys35, or both replaced by a serine residue, with the SH representing a cysteine residue and the OH a serine residue.

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but in the reduced forms of the protein. This suggestion was supported qualitatively by the destabilizing effects on the stability of the folded structure of DsbA upon replacing this accessible cysteine residue (Zapun et al., 1994) and by electrostatic calculations (Gane et al., 1995). It was further confirmed by the finding that the replacement of residues X and Y in DsbA caused changes in the pK_a values of the accessible Cys thiol group that paralleled the changes in the stability of the disulfide bond (Wunderlich, 1994; Grauschopf et al., 1995). For example, DsbA with the altered sequence -Cys-Pro-Pro-Cys-, which occurs naturally in Arabidopsis thioredoxin, has the stability of its disulfide bond increased 1700-fold and the pK_a of the first cysteine thiol group increased by 3.3 pH units (Grauschopf et al., 1995). All of these cysteine thiol groups have pK_a values lower than normal; this is believed to arise primarily from electrostatic interactions with the amide dipoles at the N-terminus of the α-helix where the cysteine residues are situated in the thioredoxin fold (Kortemme & Creighton, 1995) and to be modulated by differences in the accessibilities to solvent of these amides (Gane et al., 1995). Similar but smaller effects may be observed in simple model α-helical peptides (Kortemme & Creighton, 1995, and unpublished observations).

This correlation in DsbA between cysteine thiol ionization and stability of the disulfide bond raises the question of whether and how such a correlation is observed in other members of the thioredoxin family. It seems to be the case with the thioredoxin-like domains of PDI, which also form unstable disulfide bonds and appear to have low pK_a values for their accessible thiol groups (Darby & Creighton, 1995); it may also apply to glutaredoxin (Gan et al., 1990; Yang & Wells, 1991; Mieyal et al., 1991). If the same correlation were to apply to thioredoxin, the stability of its disulfide bond (Holmgren, 1981) would imply that its accessible cysteine thiol group have a pK_a value of about 9; it is, however, generally accepted that the actual value is about 7 (Holmgren, 1995). If the correlation does not extend to thioredoxin, the question arises as to what other aspect affects the ionization of the thiol group.

The pK_a values of thiol groups are extremely important, as they determine the ionization to the reactive thiolate anion, as well as the intrinsic reactivities of their sulfur atoms, even when converted to a disulfide bond (Creighton, 1975; Szajewski & Whitesides, 1980). Those of reduced thioredoxin (Trx_{SH}^{SH}) have been measured many times, in a number of different ways, and by several laboratories, but with somewhat different conclusions. The pH dependence of the reaction of Trx_{SH}^{SH} with thiol reagents, which generally react only with the ionized thiolate anion, was concluded to indicate a pK_a value of 6.7 for the accessible thiol group and a much higher value of about 9 for the buried thiol group (Kallis & Holmgren, 1980). Raman spectroscopy detected thiol ionization in Trx_{SH}^{SH} with an apparent pK_a of 7.5, which was attributed as the average value for the two different thiol groups: pK_a 7.1 for the accessible, but only 7.9 for the inaccessible (Li et al., 1993). NMR analysis has provided detailed information about the structures and titrations of both the dithiol and disulfide forms of *Escherichia coli* thioredoxin (Dyson et al., 1991; Jeng & Dyson, 1995) and human thioredoxin (Forman-Kay et al., 1991, 1992), but the results have been interpreted in very different ways (Wilson et al., 1995; Jeng et al., 1995; Jeng & Dyson, 1996; Qin et al., 1996). The assignment to specific groups of titrations observed by NMR is not straightforward in this case.

Very detailed structural analyses have found only minimal differences in the structures and dynamics of *E. coli* Trx_{SH}^{SH} and Trx_{S}^{S} across a wide pH range (Dyson et al., 1990, 1994; Stone et al., 1993; Chandrasekhar et al., 1994; Jeng et al., 1994; Jeng & Dyson, 1996), essentially only those required by the greater size of two thiol groups over a disulfide bond (Holmgren, 1995). Relatively few ionizations affecting the active site have been observed in either protein, yet they have been attributed very differently to the various ionizable groups of thioredoxin. The situation is believed to be complicated by linkage of ionization of the two cysteine residues to that of other ionizable groups, especially that of the buried carboxyl of Asp26, which clearly has anomalous ionization properties (Langsetmo et al., 1991a,b). At the present time, there is no consensus on the ionization properties of thioredoxin. Most of the studies have been interpreted with the constraint that one or both of the thiol groups of Trx_{SH}^{SH} must ionize in the region of pH 7.

The members of the thioredoxin family with -Cys-X-Y-Cys- active sites have very similar structures, with conservation of the ionizable residues believed to be most important (Eklund et al., 1984, 1991), so a valid view of one member should be applicable to all the others also. Our efforts to understand the catalysts of protein disulfide formation, PDI (Darby & Creighton, 1995), DsbA (Zapun et al., 1993; Nelson & Creighton, 1994), and DsbC (Zapun et al., 1995), led us to apply some of the approaches developed with these proteins to *E. coli* thioredoxin. The results obtained indicate that the active site thiol groups of thioredoxin have substantially greater pK_a values than previously thought.

EXPERIMENTAL PROCEDURES

Materials. GSH, GSSG, and DTNB were obtained from Sigma Chemical Co.; DTT was from Biomol Feinchemikallen GmbH. TFA was the sequence grade of Sigma and acetonitrile the gradient grade (LiChrosolv) of Merck. All other reagents were the highest purity commonly available. Solutions of DTT, GSH, GSSG, urea, and DTNB were prepared just before use; glutathione was neutralized with KOH. The concentration of GSH was determined by Ellman's (1959) assay, using the extinction coefficient at 412 nm of 14 150 M^{-1} cm^{-1} for the liberated NTB (Riddles et al., 1983) as described (Creighton, 1989), and that of GSSG by its extinction coefficient at 248 nm of 382 M^{-1} cm^{-1} (Chau & Nelson, 1992).

Normal thioredoxin was purchased from Promega. It was in the disulfide form, so the reduced form was prepared by incubation with DTT at 25 °C for 20 min in 0.1 M Tris-HCl, pH 7.5; the DTT was subsequently removed by gel filtration with a prepacked NAP-5 column (Pharmacia Biotech), as described by Lundström and Holmgren (1993). The *E. coli* strains harboring the plasmids that encode the active site mutant forms of thioredoxin and lacking the endogenous gene for thioredoxin, *trxA* (Russel & Model, 1986), were the gift of Dr. Marjorie Russel (The Rockefeller University, New York, NY).

Purification of the Active Site Mutants of Thioredoxin. The active site mutant forms of thioredoxin, Trx_{SH}^{OH}, Trx_{OH}^{SH}, and Trx_{OH}^{OH}, correspond to the variants with, respectively, Cys32, Cys35, and both replaced by serine residues.

They were purified with only slight modifications of the method of Russel and Model (1986), but from the proteins liberated by osmotic shock (Lunn & Pigiet, 1982) rather than the whole cell lysate. Briefly, freshly prepared cell pellet, harvested from 4 L of an overnight culture in LB broth supplemented with 10 μg/mL tetracycline, was resuspended in 160 mL of plasmolysis buffer containing 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, and 20% sucrose. After incubation at 25 °C for 10 min, the cells were collected by centrifugation, resuspended in 160 mL of distilled water prechilled on ice, and kept on ice for 10 min. After centrifugation, the proteins in the supernatant osmotic shockate were precipitated with ammonium sulfate (90% saturation). The precipitate was collected by centrifugation, resuspended in 20 mL of 20 mM Tris-HCl, pH 6.8, dialyzed against 1 L of the same buffer 3 times, and applied to a Q-Sepharose FF (Pharmacia Biotech) column (1.6 × 13.5 cm) equilibrated with the same buffer. Monitoring the elution by the absorbance at 280 nm, the column was washed at room temperature and developed with a gradient of NaCl (0−0.25 M) in 20 mM Tris-HCl, pH 6.8, over 4.2 h at a flow rate of 1 mL/min. The peak fractions containing thioredoxin, identified by electrophoretic analysis (Laemmli, 1970), were precipitated with ammonium sulfate (70% saturation). After collection by centrifugation, the pellet was dissolved in 0.6 mL of 20 mM Tris-HCl, pH 6.8, and applied at 4 °C and a flow rate of 1 mL/min to a Sephacryl S-200 HR (Pharmacia Biotech) column (1.6 × 80 cm) equilibrated with the same buffer. After the isocratic elution, the thioredoxin fractions were identified by electrophoretic analysis. For purification of Trx_{OH}^{SH}, all Tris-HCl buffers after the osmotic shock contained 5 mM DTT. The same extinction coefficient at 280 nm of 13 700 M^{-1} cm^{-1} (Holmgren & Reichard, 1967; Reutimann et al., 1981) was used to determine the concentrations of all the variants of thioredoxin.

Reactivity of the Thiol Groups of Trx_{SH}^{SH} with Iodoacetate. Trx_{SH}^{SH} at a concentration of about 5 μM was incubated with 1 mM iodoacetate at various pH values between 2 and 9 in a buffer cocktail of 50 mM each acetic acid, 2-(*N*-morpholino)ethanesulfonic acid, 3-(*N*-morpholino)propanesulfonic acid, Tris, and NH_3. After various times of reaction, the thioredoxin products were separated by the discontinuous system of Davis (1964) on the basis of their net charge, depending upon the number of acidic groups introduced by the reaction with iodoacetate. The relative rates of the reaction with iodoacetate were measured from the relative amounts of the initial and the single modified thioredoxin separated by the electrophoresis. Only thioredoxin with a single acidic group was produced in these reactions, whereas the presence of 8 M urea caused the expected species with two such modifications to be produced.

Quantification of the Dithiol and Disulfide Forms of Thioredoxin by HPLC. Trx_{SH}^{SH} and Trx_{S}^{S} were separated and quantified on a reverse phase HPLC column (Vydac 218TPS4, PROTEIN & PEPTIDE C18) at 25 °C in 0.1% TFA with a linear gradient of acetonitrile (40−60% in 20 min) at a flow rate of 1.0 mL/min. The peak corresponding to each form was determined by applying them individually and in concert. Loading the same amount of the two forms resulted in peaks of identical areas, which was then used for their quantification. To confirm that the peaks of the dithiol and disulfide forms did not overlap with those of glutathione mixed-disulfide forms, the retention times of these species were determined after generating them in the presence of 8 M urea. Reaction mixtures of 2.6 nmol of Trx_{S}^{S} equilibrated for 30 min at 25 °C with several concentrations of glutathione (6.8−20 mM GSH/0.6−5.6 mM GSSG) in 8 M urea, 0.1 M Tris-HCl, pH 8.0, were analyzed after quenching by addition of HCl to 0.3 M (see Figure 2). Mixtures expected to contain a significant amount of the three possible glutathione mixed-disulfide forms (Trx_{SH}^{SSG}, Trx_{SSG}^{SH}, Trx_{SSG}^{SSG}) showed three additional peaks. Two of the additional peaks (around 11−12 min in retention time) gave peak areas that were proportional to $[Trx_{SH}^{SH}][GSSG]/[GSH]$, indicating they are the single mixed disulfide forms, Trx_{SH}^{SSG} and Trx_{SSG}^{SH}. The other peak (around 13 min) accumulated in proportion to the GSSG concentration, consistent with it being the Trx_{SSG}^{SSG}, double mixed-disulfide species. These species were well separated from Trx_{SH}^{SH} and Trx_{S}^{S} and did not occur in the absence of urea.

Quantification of GSH and GSSG by HPLC. The amounts of GSH and GSSG present in trapped samples were determined by separating them by reverse phase HPLC in 0.1% TFA with a gradient of acetonitrile (0−15% in 15 min after holding at 0% for 10 min). Their relative peak areas were calibrated to standard solutions whose concentrations were determined by Ellman's assay for GSH and by the absorbance at 248 nm for GSSG. The analysis of the concentrations of GSH, GSSG, Trx_{SH}^{SH}, and Trx_{S}^{S} in a single reaction mixture required at most 80 min.

Estimation of the Rate and Equilibrium Constants for the Reaction Trx_{SH}^{SH} + GSSG ↔ Trx_{S}^{S} + 2GSH. Trx_{S}^{S} or Trx_{SH}^{SH} at a concentration of 5 μM was incubated at 25 °C with a solution containing GSH or GSSG, respectively, plus 0.2 M KCl and 1 mM EDTA, and buffered with either 0.1 M citrate (for pH 4.2−5.2), 0.1 M phosphate (for pH 6.3−6.8), 0.1 M Tris-HCl (for pH 7.1−8.7), or 0.1 M glycine−NaOH (for pH 9.6−10.3). After various time periods, the reaction was quenched by addition of HCl to 0.3 M. The relative concentrations of Trx_{S}^{S} and Trx_{SH}^{SH} were determined by HPLC analysis as described above. The oxidation rate constant for formation of Trx_{S}^{S} was calculated by fitting with a nonlinear least-squares procedure the change in the concentration of the initial fraction of Trx_{SH}^{SH} ($F_{initial}$) with time (t) to an exponential function:

$$F_{initial}(t) = F_{initial}(\infty) + A \exp(-k_{ox}[GSSG]t) \quad (1)$$

where k_{ox} is the oxidation rate constant. The reverse rate constant, k_{red}, was estimated by another fitting procedure because of significant reversal of the reaction by the small amounts of GSSG present in the initial preparation of GSH. The fraction of Trx_{S}^{S} ($F_{initial}$) with time (t) was fitted to an exponential:

$$F_{initial}(t) = \frac{(k_{red}[GSH]^2 \exp\{-(k_{ox}[GSSG] + k_{red}[GSH]^2)t\} + k_{ox}[GSSG]}{k_{ox}[GSSG] + k_{red}[GSH]^2} \quad (2)$$

For determination of the equilibrium constant K_{GSSG}, Trx_{S}^{S} was incubated with various concentrations of GSSG and GSH (6 combinations for each pH) in the same way as

described in the former section. After 90 min incubation at 25 °C, the solution was quenched with 0.3 M HCl. This incubation period was determined to be sufficient to reach equilibrium at pH values greater than 6.3 (data not shown). An aliquot containing 0.5 nmol of thioredoxin was then analyzed by HPLC for the relative concentrations of Trx_{SH}^{SH}, Trx_{S}^{S}, and, when necessary, GSSG and GSH. The value of the equilibrium constant, K_{GSSG}, was estimated by a linear least-squares fitting of the ratio of $[Trx_{SH}^{SH}]/[Trx_{S}^{S}]$ as a function of $[GSH]^2/[GSSG]$ in eq 6. At pH less than 6.3, equilibrium would have required substantially longer periods of incubation, so the equilibrium constant was determined kinetically by the ratio of the forward and reverse rate constants as described above.

Kinetics of the Reaction of the Thioredoxin Thiol Groups with DTNB. Ellman's reaction (Riddles et al., 1983) was performed with the thioredoxin mutants, Trx_{SH}^{OH}, Trx_{OH}^{SH}, and Trx_{OH}^{OH}, plus wild-type protein, Trx_{SH}^{SH}. After treatment with DTT and gel filtration as described in the preceding section, the thioredoxin proteins were mixed with a 0.9 volume of 10 mM DTNB in the gel filtration buffer (pH 5.0) or 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, 0.1 mM EDTA, including 0.1 volume of the gel filtration buffer in a cuvette with 1.0 cm light path. The reaction at 25 °C was followed by monitoring the absorbance at 412 nm with the Kontron spectrophotometer. Preceding the addition of DTNB, the absorbance at 280 nm of each solution was measured to determine its thioredoxin concentration.

Estimation of the Rate and Equilibrium Constants for the Reaction $Trx_{OH}^{SH} + GSSG \leftrightarrow Trx_{OH}^{SSG} + GSH$. The procedures were analogous to those used with normal thioredoxin described above. When 1−2 μM Trx_{OH}^{SH} was reacted with 1−5 mM GSSG, significant amounts of a species believed to be thioredoxin disulfide dimer were generated, complicating the analysis. This species did not appear during the reverse reaction, between Trx_{OH}^{SSG} and GSH, and both the forward and reverse rate constants could be obtained from the kinetics of approach to equilibrium of the reaction, as in Figures 1 and 3 for the normal protein. The species Trx_{OH}^{SH} and Trx_{OH}^{SSG} had virtually the same chromatographic properties as the normal species Trx_{SH}^{SH} and Trx_{SH}^{SSG}, as expected.

Measurement of UV Absorbance Spectra. To ensure that the cysteine residues were in the thiol form, the thioredoxin variants possessing cysteine residues were incubated for 20 min at 25 °C in 10 mM DTT, 0.1 M Tris-HCl, pH 7.5. They were gel-filtered through prepacked NAP-5 columns (Lundström & Holmgren, 1993) in a buffer of 1 mM citrate, 1 mM borate, 1 mM phosphate, 0.2 M KCl, and 0.1 mM EDTA, pH 5.0. In this buffer, normal thioredoxin, Trx_{SH}^{SH}, remained reduced for at least 2 days according to the HPLC analysis described in the following section. The reduced protein solution was mixed with 9 volumes of buffer solutions consisting of 0.2 M KCl and 1 mM each of phosphate, citrate, and borate, adjusted to the appropriate pH with NaOH and HCl; the total concentrations of Na^+ and Cl^- were kept at 0.1 M for constant ionic strength. Absorbance spectra were measured with thioredoxin concentrations of 3−10 μM in a cuvette with 1.0 cm light path at 25 °C. Spectra were taken in 0.5 nm steps from 370 to 240 nm, using a Uvicon 930 spectrophotometer (Kontron) equipped with cuvette holder thermostated at 25 °C with circulating water. Immediately after each spectrophotometric measurement, the pH value of the solution was measured using a Radiometer PHM 83 pH meter. The spectrum for each protein was subtracted from that of the buffer without protein in the same cuvette. As each variant had the same absorbance at 280 nm in a series of buffer solutions with different pH values, all spectra were converted to molar extinction coefficients on the basis of the absorbance at 280 nm.

RESULTS

Chemical Reactivity of the Thiol and Disulfide Groups of Thioredoxin. Virtually all chemical reactions of thiol groups occur with the ionized thiolate anion, not with the nonionized thiol form, so the variation with pH of the reactivity of a thiol group is generally considered to be a valid measure of its pK_a value. Kallis and Holmgren (1980) demonstrated that the total rate of reaction of Trx_{SH}^{SH} with iodoacetamide or iodoacetate was maximal at alkaline pH, decreased at about pH 9 to a somewhat lower rate of reaction, and then decreased further at about pH 6.7 to an even lower rate of reaction. The groups titrating at pH 9 and 6.7 were interpreted to be Cys35 and Cys32, respectively. If this were the case, however, the rate of reaction should approach zero at lower pH values, but it did not, which could be interpreted as the thiolate anion being present even at very low pH values.

These results were confirmed here with iodoacetate, but extended to lower pH values than the lowest value of about 5.7 measured by Kallis and Holmgren (1980) (data not shown). The reaction was followed electrophoretically by the charge change introduced into thioredoxin by the reaction with acidic iodoacetate, which also demonstrated that only one cysteine thiol group reacted over the entire pH range of 2−9. The pH dependence of the reactivity was very similar to that reported by Kallis and Holmgren (1980), and the significant rate of reaction at pH 5.7 was observed to be maintained at lower pH values; it became negligible only below pH 3, where Trx_{SH}^{SH} unfolds (Langsetmo et al., 1989). Thioredoxin is known to have minimum solubility near its isoelectric point of pH 4.5 (Holmgren, 1985; Hiraoki et al., 1988; Dyson et al., 1991), but there were no indications of precipitation or aggregation at the low concentrations used in any of the studies presented here (e.g., Laurent et al., 1964; Ladbury et al., 1993).

These data indicate that titrations at pH values of about 3, 7, and 9 affect the reactivity of the Cys32 thiol group of Trx_{SH}^{SH}, but do not indicate which correspond to ionization of the thiol group itself. The pH dependence of the rate of alkylation of Trx_{SH}^{SH} is remarkably similar to that of DsbA, where the thiol ionization occurs with an apparent pK_a of about 3.5 (Nelson & Creighton, 1994). The very similar data for thioredoxin might be indicating a similar, very low pK_a value for Cys32, but further data are necessary.

The thiol−disulfide exchange reaction between Trx_{SH}^{SH} and GSSG, to form Trx_{S}^{S}:

$$Trx_{SH}^{SH} \underset{K_1}{\overset{GSSG \quad GSH}{\rightleftharpoons}} Trx_{SH}^{SSG} \underset{K_2}{\overset{GSH}{\rightleftharpoons}} Trx_{S}^{S} \qquad (3)$$

$$K_1 = \frac{k_{+1}}{k_{-1}} = \frac{[\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SSG}}][\mathrm{GSH}]}{[\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}][\mathrm{GSSG}]} \quad (4)$$

$$K_2 = \frac{k_{+2}}{k_{-2}} = \frac{[\mathrm{Trx}_{\mathrm{S}}^{\mathrm{S}}][\mathrm{GSH}]}{[\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SSG}}]} \quad (5)$$

$$K_{\mathrm{GSSG}} = \frac{k_{\mathrm{ox}}}{k_{\mathrm{red}}} = K_1 K_2 = \frac{[\mathrm{Trx}_{\mathrm{S}}^{\mathrm{S}}][\mathrm{GSH}]^2}{[\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}][\mathrm{GSSG}]} \quad (6)$$

was also measured. The thiol−disulfide exchange reaction is very specific and is well understood (Szajewski & Whitesides, 1980). The reaction can be rapidly quenched by acidification and monitored by HPLC separation of the various trapped reactants, intermediates, and products.

The observed rate of the reaction between $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}$ and GSSG is expected to be determined by the initial reaction of the accessible thiol group of $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}$ with GSSG, as the second, intramolecular step in $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SSG}}$ of forming the disulfide bond should be very rapid (k_{+1} [GSH] $\ll k_{+2}$) and the reverse reactions should be negligible in the absence of GSH. In agreement, the intermediate $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SSG}}$ was not apparent, and only the initial and final species, $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}$ and $\mathrm{Trx}_{\mathrm{S}}^{\mathrm{S}}$, were observed throughout the time course of the reaction in both directions (Figure 1A,B). To ensure that $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SSG}}$ and any other mixed disulfides of glutathione with the protein would have been detected, the same reaction was carried out in 8 M urea, where $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}$ is unfolded; consequently, its two cysteine residues show comparable reactivities, and both accumulate as mixed disulfides with glutathione (Figure 2). This demonstrated that the mixed-disulfide species would have been detected but were not trapped at significant concentrations in the absence of urea (Figure 1). Therefore, the rate of the forward reaction of eq 3 is given by the initial reaction between GSSG and the accessible thiol group of $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}$, k_{+1}.

When the pH was varied between 4 and 11, the value of k_{+1} was found to be essentially constant above pH 8 and to decrease at lower pH values, down to nearly pH 4, approximately as expected for a thiol group with a pK_a value of about 7.0 (Figure 3A). There were no indications of substantial changes in rate due to ionizations in the regions of pH 3 and 9, as observed by the reaction with iodoacetate or iodoacetamide described above. This pH dependence contrasts with that observed with DsbA, where the rate was virtually independent of pH down to pH 4, presumably due to full ionization of its accessible thiol group at all such pH values (Nelson & Creighton, 1994).

The rate of the reverse reaction of eq 3, between $\mathrm{Trx}_{\mathrm{S}}^{\mathrm{S}}$ and 2GSH, was measured in the same way (Figure 1B). In this case, the high stability of the disulfide bond of $\mathrm{Trx}_{\mathrm{S}}^{\mathrm{S}}$ (Holmgren, 1981; Lin & Kim, 1989) resulted in its reduction being reversed rapidly by the small amounts of GSSG contaminating the GSH. $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}$ was generated only partially at equilibrium (Figure 1C). The actual concentrations of GSH and GSSG were determined, so the rate constants for both reduction and oxidation could be obtained from such experiments by simulation of the time course of the reaction. The rate constants for the forward reaction obtained in this way were very similar to those measured directly (Figure 3A).

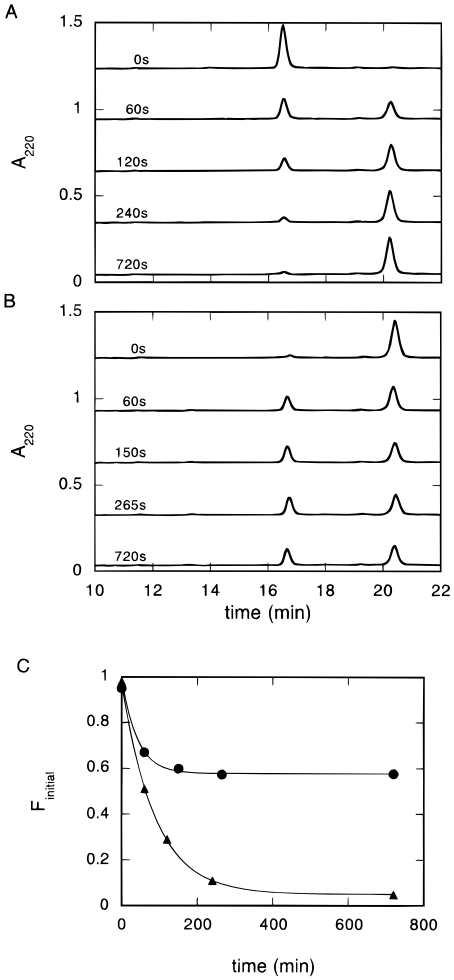

FIGURE 1: Kinetics of the reaction of $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}$ + GSSG (A) and of $\mathrm{Trx}_{\mathrm{S}}^{\mathrm{S}}$ + 2GSH (B) monitored by HPLC separation of the acid-trapped species of thioredoxin. In each case, 5.0 μM $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}$ or $\mathrm{Trx}_{\mathrm{S}}^{\mathrm{S}}$ was incubated at pH 8.7 with either 68.3 μM GSSG or 27.0 mM GSH (but also containing 93.6 μM GSSG initially), respectively, for the indicated time period, trapped by acidification, and a 0.60 nmol portion subjected to HPLC analysis; the HPLC profiles depicting the absorbance at 220 nm (A_{220}) are offset vertically for clarity. The fractional amounts of the initial species, $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}$ (triangles) and $\mathrm{Trx}_{\mathrm{S}}^{\mathrm{S}}$ (circles), for the two reactions are plotted as a function of time in panel C. The apparent rate constants were estimated from these data by a least-squares procedure to be 153.0 s^{-1} M^{-1} (triangles) and 14.4 s^{-1} M^{-2} (circles); the predicted time courses are shown in panel C by the solid curves.

The measured rate constants for the reaction between $\mathrm{Trx}_{\mathrm{S}}^{\mathrm{S}}$ and GSH varied with pH in a manner very similar to that observed for the other direction (Figure 3A), decreasing continuously below pH 7 and being relatively constant above that pH. The rate of this reaction was proportional to [GSH]^2 and is expected to depend upon two factors: (1) the unfavorable equilibrium of the reaction between $\mathrm{Trx}_{\mathrm{S}}^{\mathrm{S}}$ and GSH to generate the unstable $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SSG}}$, $1/K_2$ of eq 5, which will be reversed rapidly and intramolecularly; and (2) the rate of reaction of the intermediate $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SSG}}$ with GSH to generate $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SH}}$ and GSSG, k_{-1} of eq 4. The observed rate constant will be k_{-1}/K_2. Unfortunately, the mixed-disulfide $\mathrm{Trx}_{\mathrm{SH}}^{\mathrm{SSG}}$ was not present at detectable concentrations, so the values of neither k_{-1} nor K_2 could be measured directly.

The value of K_2 could be measured indirectly in the case of the mutant form of thioredoxin lacking Cys35, $\mathrm{Trx}_{\mathrm{OH}}^{\mathrm{SH}}$, as

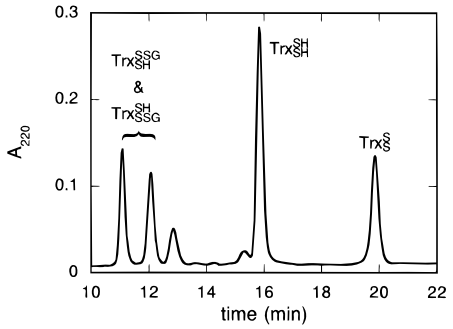

FIGURE 2: Separation of the thiol/disulfide species of thioredoxin by reverse phase HPLC. Trx_S^S (2.6 nmol) was equilibrated with 10 mM GSH and 1.21 mM GSSG in 8 M urea, 0.1 M Tris-HCl, pH 8.0, for 30 min at 25 °C. After quenching with 0.3 M HCl, portions were analyzed by reverse phase HPLC, monitoring the absorbance at 220 nm (A_{220}). The separation used a linear gradient of acetonitrile (40−60% v/v in 20 min) in 0.1% TFA at 25 °C and a flow rate of 1.0 mL/min. The void volume of the entire system was about 5 mL. The peak eluting just before 13 min is believed to be Trx_{SSG}^{SSG}.

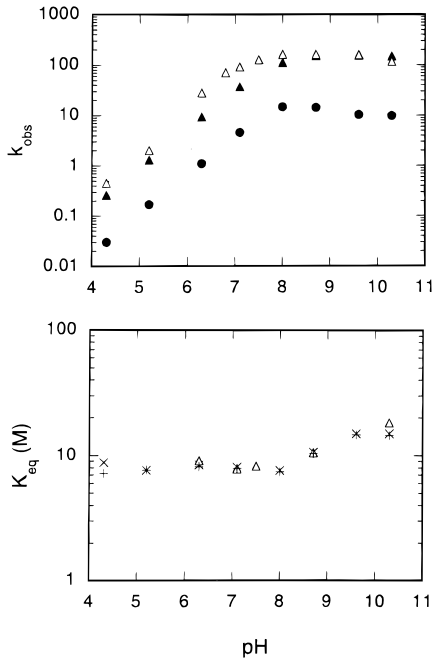

FIGURE 3: pH dependence of the rate and equilibrium constants for the thiol−disulfide exchange reaction between thioredoxin and glutathione. (Top, A) The measured rate constants for the reactions of Trx_{SH}^{SH} + GSSG (s^{-1} M^{-1}, triangles) and of Trx_S^S + 2GSH (s^{-1} M^{-2}, circles) were estimated as in Figure 1 from HPLC quantification of the species generated during the former and latter reactions (open and closed symbols, respectively). (Bottom, B) The value of the equilibrium constant (in units of M) was estimated by three different methods: (1) When equilibrium was reached within 90 min, the equilibrium constant was calculated directly (eq 6) from the ratios of Trx_{SH}^{SH} and Trx_S^S measured at equilibrium with various concentrations (at least six combinations) of GSH and GSSG (triangles). (2) The value of K_{GSSG} was calculated by the ratio of the rate constants for both directions measured in (A) from the kinetics of reaction between Trx_S^S and 2GSH (A) (×). (3) From the time course of the same experiments, the ratios of $[Trx_{SH}^{SH}]/[Trx_S^S]$ at equilibrium were estimated by extrapolation of the time course with single-exponential least-squares fitting (+).

only Trx_{OH}^{SSG} was generated by the reaction with GSSG. That data for K_1 plus the pH independence of K_{GSSG} (see Figures 3B and 5 below) indicate that the value of K_2 is also independent of pH over the range of pH 4−10. Therefore, the pH dependence of the observed rate of reduction of Trx_S^S by GSH is that of k_{-1}, which involves the reaction of the thiol group of GSH. The data were consistent with a pK_a value of about 7, although the rate decreased at lower pH values somewhat less than expected; the slope of the plot of Figure 3A at low pH values is about 0.9, rather than the expected 1.0. The apparent pK_a of 7 indicated by these data contrasts with the known value of 8.8 for the thiol group of GSH (Jung et al., 1972; Chau & Nelson, 1991).

The various and inconsistent results obtained kinetically by the reaction of thioredoxin with iodoacetamide, iodoacetate, GSSG, and GSH suggest that the rates of these reactions are not reflecting primarily the proportion of thiolate anion in the reactive thiol species.

pH Dependence of the Equilibrium between Thioredoxin and Glutathione. The kinetic data of Figure 3A, in which the observed rate constants in both directions varied similarly with pH, imply that the overall equilibrium for the reaction between thioredoxin and glutathione of eq 3, K_{GSSG}, should be nearly independent of pH. This was confirmed directly with equilibrium mixtures, analyzed with the same techniques used for the kinetic experiments (Figure 3B). The values measured here are consistent with those measured by others at individual pH values (Holmgren, 1981; Lin & Kim, 1989).

The value of the apparent equilibrium constant was virtually constant between pH 4 and 8 and only increased 2-fold at higher pH values. This result is very important, for if the thiol groups of reactants and products, but no others, differ in their ionization properties at any pH values, the equilibrium constant for that reaction must change in that pH region. In general, a plot of log K_{eq} *versus* pH will have a slope equal to the net number of protons liberated in the reaction. Other than the thiol group, GSH and GSSG do not differ substantially in their ionization properties (Jung et al., 1972), so the ionization properties of the protein will be those most important for determining the pH dependence of the equilibrium between thioredoxin and glutathione (eq 3). Considering only thiol groups, the equilibrium will depend upon the relative pK_a values of the two thiol groups of Trx_{SH}^{SH} and those of the two GSH molecules generated by eq 3. If either of the reactant thiol groups of Trx_{SH}^{SH} had a lower pK value than the product thiol group of GSH, the equilibrium constant should increase by a factor of 10 for each unit decrease in pH within the region between the two pK_a values. This was observed with DsbA, where the value of K_{GSSG} increased by 3 orders of magnitude between pH 9 and 4, and suggested only one linkage in ionization of about 1 pH unit (Nelson & Creighton, 1994).

The simplest interpretation of the data of Figure 3B would be that the two thiol groups of Trx_{SH}^{SH} have pK_a values somewhat greater than that of GSH, 8.8, and that no other groups have markedly different pK_a values in Trx_{SH}^{SH} and Trx_S^S. If, however, Trx_{SH}^{SH} and Trx_S^S do differ in the ionization of other groups, this result would require that the net charge of Trx_{SH}^{SH} + GSSG not differ substantially from that of Trx_S^S + 2GSH throughout the entire pH region; this places limitations on the possibilities (see Discussion).

Reactivity of the Individual Thiol Groups of Thioredoxin. Only the accessible thiol group of Cys32 of Trx_{SH}^{SH} is reactive to thiol reagents (Takahashi & Hirose, 1990), and this was confirmed here. Even at alkaline pH values of 8.7 and 9, only one thiol group was found to be reactive to

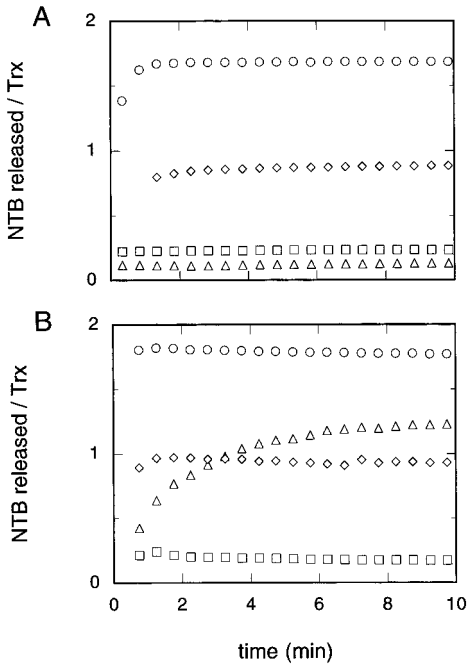

FIGURE 4: Reaction of the thiol groups of thioredoxin with DTNB. The reaction of Trx^{SH}_{SH} (circles), Trx^{OH}_{SH} (triangles), Trx^{SH}_{OH} (diamonds), or Trx^{OH}_{OH} (squares) with 1 mM DTNB at 25 °C and either pH 5.0 (A) or pH 7.5 (B) was monitored by the absorption increase due to liberation of NTB.

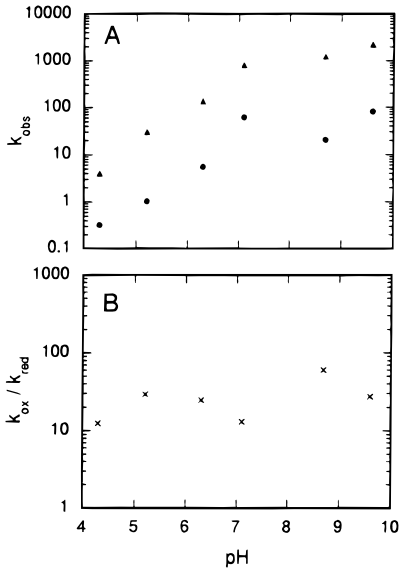

FIGURE 5: pH dependence of the rate and equilibrium constants for the thiol−disulfide exchange reaction between Trx^{SH}_{OH} and glutathione. (A) The rate constants (s^{-1} M^{-1}) for the reactions of Trx^{SH}_{OH} + GSSG (k_{+1}, triangles) and of Trx^{SSG}_{OH} + GSH (k_{-1}, circles) were estimated from HPLC quantification of the species generated during the reaction between Trx^{SSG}_{OH} and GSH, as in Figure 3A. (B) The value of the equilibrium constant was estimated by the ratio of the two rate constants.

iodoacetamide or iodoacetate, using electrophoretic and mass spectrometry methods (data not shown). The increased reactivity above pH 8.5 observed by Kallis and Holmgren (1980) therefore is unlikely to have come from the contribution of the buried Cys thiol group to the reaction, as had been assumed. The lack of reactivity of the totally buried thiol group of Cys35 is not surprising on structural grounds (Jeng et al., 1994), and it would probably also be diminished in the above experiments by the more rapid and prior reaction of the accessible thiol group of Cys32. The ionizations of the two thiol groups are very likely to be linked, as has been shown in DsbA (Zapun et al., 1994; Nelson & Creighton, 1994).

The intrinsic reactivities of the ionized thiol groups of Trx^{SH}_{SH} are studied more directly in mutant forms in which the other cysteine residue has been replaced by serine (Russel & Model, 1986): Trx^{SH}_{OH}, Trx^{OH}_{SH}, and Trx^{OH}_{OH}. The structure of the double mutant Trx^{OH}_{OH} is very similar to that of Trx^{SH}_{SH} (Dyson et al., 1994), indicating that these are suitable models for the normal protein. Their reactivities to the disulfide reagent DTNB (Ellman, 1959) were investigated at both neutral and acidic pH, 7.5 and 5.0, respectively. This reagent is analogous to GSSG, and is expected to react with Trx^{SH}_{SH} in the same way (eq 3), but it liberates the colored thiol NTB, which is measured. As expected, DTNB did not react with Trx^{OH}_{OH}, but did with Trx^{SH}_{SH}, too rapidly to measure at both pH 5.0 and 7.5, to produce 2 mole of NTB per mol of protein (Figure 4). The accessible thiol group of Trx^{SH}_{OH} also reacted very rapidly at both pH values and liberated only 1 mol of NTB, as expected. The buried thiol group of Trx^{OH}_{SH} reacted only slowly at pH 7.5 and not detectably at pH 5.0. Although only qualitative, these data confirm that the thiol group of Trx^{OH}_{SH} is likely to have a pK_a value considerably greater than 7.5.

The reactivity of the single, accessible thiol group of Trx^{SH}_{OH} with GSSG, to form the mixed-disulfide Trx^{SSG}_{OH}, was measured using the same procedures as with the normal protein (Figures 1−3). Both the rates and equilibrium were measured over the pH range 4−10 (Figure 5). The pH dependence of the rate of reaction between Trx^{SH}_{OH} and GSSG, k_{+1}, was very similar to that observed with the normal protein, as expected, although the rate constant was uniformly 10-fold greater. The reverse rate constant, k_{-1}, had a similar pH dependence. As expected, the value of the equilibrium constant, K_1, was independent of pH. The pH independence of the values of both K_1 and K_{GSSG} implies that K_2 is also independent of pH over the same range (eq 6).

The pH independence of K_1 suggests that the pK_a of the thiol group of Trx^{SH}_{OH} is not less than that of GSH, 8.8.

Measurement of the Ionization of Active Site Thiols by UV Absorbance. The thiolate anion has a significant absorption at around 240 nm, whereas the protonated thiol does not, so this absorbance is a most direct measure of the ionization and pK_a value of a thiol group (Benesch & Benesch, 1955; Polgar, 1974). With proteins, it requires careful subtraction of spectral changes from other titrating groups and from any pH-dependent conformational changes, usually by comparison to the same protein without the cysteine thiol group (either modified covalently or replaced mutagenically). The technique has given valid data with papain (Polgar, 1974), glutathione transferase (Graminski et al., 1989; LoBello et al., 1993), and DsbA (Nelson & Creighton, 1994), so it was of interest to apply it to thioredoxin.

In the present investigation, the absorbance properties of Trx^{SH}_{SH}, Trx^{OH}_{SH}, Trx^{SH}_{OH}, and Trx^{OH}_{OH} were compared (Figure 6). Their absolute absorbance values at 240 nm are presented in Figure 6A. Their absorbances at 288 and 295 nm were also monitored as probes of unfolding and of ionization of

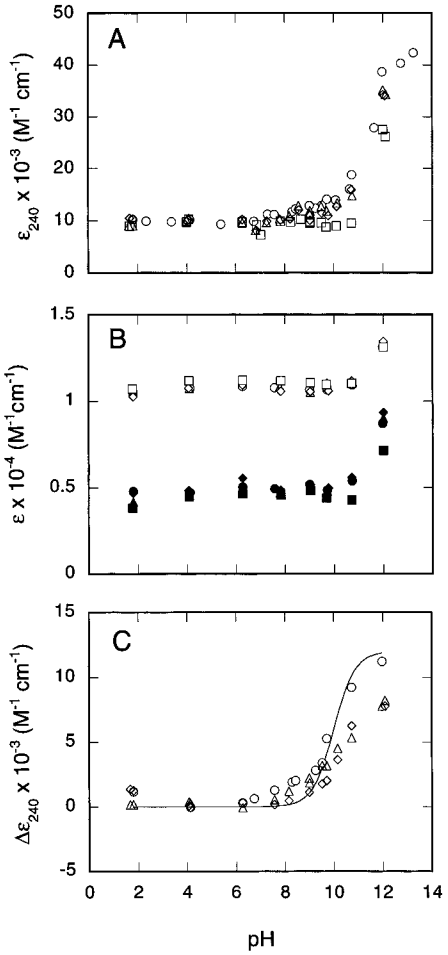

FIGURE 6: Measurement of thiol ionization in thioredoxin variants by ultraviolet absorbance. The pH dependence of the molar extinction coefficients of Trx_{SH}^{SH} (circles), Trx_{SH}^{OH} (triangles), Trx_{OH}^{SH} (diamonds), and Trx_{OH}^{OH} (squares) is given in panel A for 240 nm and in panel B for 288 nm (open symbols) and 295 nm (closed symbols). The differences in the absorbance at 240 nm between that of Trx_{OH}^{OH} and either Trx_{SH}^{SH} (circles), Trx_{SH}^{OH} (triangles), or Trx_{OH}^{SH} (diamonds) are given in panel C. The solid curve there gives the theoretical titration curve expected for a simple group with a single and constant pK_a value. The data of two independent measurements are superimposed in each case.

tyrosine residues, respectively (Figure 6B). All the variants showed similar behavior at 288 nm, indicating that they had unchanged conformations below pH 11, but they began to denature at higher pH (Figure 6B). Dyson et al. (1991) have shown by NMR analysis that both Trx_{SH}^{SH} and Trx_S^S remain folded up to pH 10. The spectra of the variants possessing cysteine residue(s), Trx_{SH}^{SH}, Trx_{SH}^{OH}, and Trx_{OH}^{SH}, were subtracted from that of the control, Trx_{OH}^{OH}, and the absorbance differences at 240 nm are shown in Figure 6C. Similar results were obtained at pH values less than 10 using the disulfide form Trx_S^S as the control without thiol groups (data not shown), but it unfolded only at higher pH values than the other forms of the protein, as expected, and therefore was not an adequate control over the entire pH range.

Each of the variants with thiol groups appeared from its absorbance spectrum to exhibit full ionization of its thiol groups by pH 11, just prior to unfolding of the protein. For both Trx_{SH}^{OH} and Trx_{OH}^{SH}, the magnitude of the increase of ϵ_{240} at pH 11 over that at low pH was close to the expected value of 5×10^3 M^{-1} cm^{-1}, the molar extinction coefficient of a single cysteine residue. For Trx_{SH}^{SH}, the change was approximately double, as expected for ionization of two cysteine residues.

None of the thioredoxin variants with thiol groups showed evidence of thiol ionization below pH 7. In each case, there was a slight increase in the absorbance at 240 nm between pH 7 and 9, but corresponding to no more than about 20% ionized form of one thiol group. The major appearance of the thiolate anion occurred only above pH 9. Fitting by a nonlinear least-squares procedure gave pK_as of 9.9, 9.9, and 10.3 for the major transitions of Trx_{SH}^{SH}, Trx_{SH}^{OH}, and Trx_{OH}^{SH}, respectively. These pK_a values cannot be considered very accurate, however, due to the complications of tyrosine ionization and protein unfolding that occur at such high pH values and affect the absorbance spectra (Figure 6B).

DISCUSSION

The ionization properties of thioredoxin in its dithiol and disulfide states have been studied in great detail by several laboratories, using some of the most sophisticated techniques, yet there is no consensus on the interpretation of even identical results (e.g., Wilson et al., 1995; Jeng et al., 1995). The primary reason for studying this contentious problem was to extend and test our current understanding of other members of the thioredoxin family, namely, DsbA and the thioredoxin-like domains of PDI (Darby & Creighton, 1995). There the situation seems clear: these proteins have oxidative functions and therefore have relatively unstable disulfide bonds at their active sites that can be readily transferred to their target molecules. In contrast, thioredoxin functions as a reductant and, as expected, forms a much more stable disulfide bond (Holmgren, 1981, 1985, 1995). The disulfide instability of DsbA and PDI arises indirectly due to stabilization of the dithiol form of the protein, primarily due to favorable electrostatic interactions of amide groups at the N-terminus of an α-helix in the folded state with the thiolate anion of the accessible active site cysteine residue (Nelson & Creighton, 1994; Kortemme & Creighton, 1995). Increasing the pK_a of the accessible thiol group of DsbA increases the stability of its disulfide bond (Grauschopf et al., 1995). This relationship would be expected to hold with thioredoxin also, for the ionizable residues in the active sites of all these proteins are closely conserved (Eklund et al., 1984, 1991) and the structures of the disulfide bonds of DsbA and Trx_S^S are indistinguishable crystallographically (Martin et al., 1993). In this case, the thiol group of Trx_{SH}^{SH} would be expected to have a relatively high pK_a value of about 9, and the data presented here confirm this expectation. Yet this conclusion is at variance with the current consensus that the pK_a value is considerably lower, in the region of 7.

In assessing the available data on thioredoxin, a number of considerations must be kept in mind. The ionization of each group in a folded protein will be affected to varying extents by the ionization of all other groups in the protein, and the effective pK_a of a group can change with pH, even when only a single major ionization of that group is observed. If any other properties of the various ionization states of the protein differ, such as solubility or stability of the folded state, such parameters will affect the apparent pK_a value. In the case of the thioredoxin family, this extends to the equilibrium for forming the active site disulfide bond, because thiol groups ionize whereas disulfide bonds do not.

Furthermore, Trx_{SH}^{SH} and Trx_{S}^{S} are known to differ in their (a) stabilities to unfolding (Holmgren, 1985; Kelley et al., 1987; Hiraoki et al., 1988; Lin & Kim, 1989), (b) *cis-trans* isomerization of the peptide bond between Ile75 and Pro76 (Langsetmo et al., 1989), (c) aggregation properties (Ladbury et al., 1994), (d) partial specific volume (Kaminsky & Richards, 1992), and (e) possibly the ionization of other residues, particularly Asp26 (Wilson et al., 1995). If there are large interactions between ionizable groups, a single proton can be shared among them, as occurs in thiol proteases (Polgar, 1974; Lewis et al., 1981) and has been proposed for the thiol groups of Trx_{SH}^{SH} (Jeng et al., 1995). In such a case, the ionization properties of the entire system of linked groups must be considered, not just single pK_a values of individual groups.

Fortunately, the linkage of ionization of groups can be used in reverse to uncover such linkages, for the effects must be exactly reciprocal: if process X alters the ionization of group Y by *i* pH units (altering the ionization equilibrium by a factor of 10^i), the ionization of group Y must alter process X by the same factor of 10^i. Such effects are well documented in DsbA. (1) The native conformation of DsbA destabilizes its disulfide bond, which is more stable in the unfolded protein, so the disulfide bond destabilizes the folded conformation to about the same extent (Zapun et al., 1993; Wunderlich et al., 1993). The much more stable disulfide bond of Trx_{S}^{S} stabilizes the folded conformation to about the expected extent (Kelley et al., 1987; Lin & Kim, 1989). The effect of a disulfide bond on the stability of a folded conformation can be used to estimate the stability of that disulfide bond (Creighton, 1986). (2) The native conformation of DsbA stabilizes the thiolate anion of its active site cysteine residue, so the thiolate anion should stabilize the native conformation. In accord, replacing this cysteine residue by a neutral residue, or involving it in a disulfide bond, decreases the stability of the native conformation (Zapun et al., 1994). The magnitudes of the effects are not the same, however, probably due to the sensitivity of the active site to mutation (Grauschoph et al., 1995). The effects of replacing ionizable groups on the overall stability of the folded conformation can be used to estimate the relative pK_a of that group in the folded and unfolded states (Tanford, 1970). (3) If the ionization of group A affects the ionization of group B, the ionization of group B must have the same effect on the pK_a of group A. If the ionization of thiol groups is affected by ionization of other groups, the ionization of these groups must also be affected by the ionization of the thiol groups, or by their replacement by mutation, chemical modification, or disulfide formation.

A crucial question with thioredoxin regards the degree of linkage of ionization of its thiol groups to the ionization of other groups of the protein. If there are large effects, as has often been assumed, the ionization of these other groups must be affected to the same extent by the titration or removal of the thiol groups. Yet detailed NMR analysis (Dyson et al., 1990, 1991, 1994; Jeng et al., 1994) has found no substantial structural changes in Trx_{SH}^{SH} and Trx_{S}^{S} (Holmgren, 1995) and very few differences between them. Major ionizations occur at about pH 7.5 in Trx_{S}^{S} (Dyson et al., 1991), with an additional titration in Trx_{SH}^{SH} at about pH 8.4 (Dyson et al., 1991) or pH 9.5 (Wilson et al., 1995). Most protons of the active site of Trx_{SH}^{SH} and Trx_{S}^{S} are affected only by titrations with these pK_a values (Dyson et al., 1991). The groups titrating with the same pK_a value in Trx_{SH}^{SH} and Trx_{S}^{S} need not be the same, of course, but it is necessary to assume a coincidence of pK_a values if there are large pK_a effects linked to the thiol group ionizations.

A prime candidate for a group that would affect the ionization of the thiol groups of Trx_{SH}^{SH} is Asp26, which is close to Cys35 and largely buried in the folded conformation (although interacting with the amino group of a lysine residue). Replacing this residue by alanine substantially stabilizes the folded conformation in a pH-dependent manner, consistent with it having a greatly elevated pK_a value (Langsetmo et al., 1990, 1991a,b; Ladbury et al., 1993). But its pK_a values in Trx_{SH}^{SH} and Trx_{S}^{S} are very uncertain. Similar pK_a values of 7.1−7.5 have been assigned in Trx_{SH}^{SH} and Trx_{S}^{S} (Dyson et al. 1991; Jeng et al., 1995; Jeng & Dyson, 1996), whereas Wilson et al. (1995) and Qin et al. (1996) believe that the value is >9 in Trx_{SH}^{SH} and 7.5 in Trx_{S}^{S}. In this latter case, ionization of Asp26 would be strongly linked to thiol ionization and to the stability of the disulfide bond; therefore, replacing Asp26 would be expected to alter both parameters. Yet Gleason (1992) has reported that the change of Asp26 to Ala causes no change in the relative stability of the disulfide bond at pH 7. This measurement needs to be extended to more basic pH values, but it suggests that Asp26 is not involved in regulating the stability of the disulfide bond of Trx_{S}^{S}. Asp26 is probably the only group in Trx_{S}^{S} to have anomalous titration properties, as the stability of the Ala26 protein was independent of pH between 7 and 8.5 (Langsetmo et al., 1991a). The acidic Asp26 is not unique to thioredoxin, for DsbA and the PDI domains have a corresponding Glu residue.

Most of the complications in the interpretation of these titration data arise because of the expectation that at least one of the thiol groups of Trx_{SH}^{SH} is titrating at about pH 7.5 (Wilson et al., 1995). In the absence of this assumption, a simpler interpretation would be that the same groups are titrating at pH 7.5 in Trx_{SH}^{SH} and Trx_{S}^{S} and that the group titrating at pH 9.5 in Trx_{SH}^{SH} is the accessible thiol group, as indicated by the data presented here. This alternative explanation has not been ruled out. Indeed, it is supported by the absence of substantial effects on the structure and pH titration behavior of the protein in which both thiol groups have been replaced by hydroxyls, Trx_{OH}^{OH} (Jeng et al., 1994); the absence of the thiol groups should alter the ionization of any linked groups.

The major evidence for a relatively low pK_a value of a thiol group of Trx_{SH}^{SH} has been the pH dependence of the rate of reaction with iodoacetamide and iodoacetate, which was interpreted as indicating a pK_a of 6.7 for Cys32 and about 9 for Cys35 when Cys32 had already reacted (Kallis & Holmgren, 1980). The experimental observations have been confirmed here, but only Cys32 has been found to be reactive [see also Takahashi and Hirose (1990)], and the substantial reactivity at very low pH values has been confirmed and extended. The data therefore indicate that multiple ionizations affect the reactivity of Cys32, with apparent pK_a values of about 3, 6.7, and about 9, and it is not clear which would correspond to the major thiol ionization. It is remarkable that very similar rates of reaction with iodoacetate and their dependence on pH were observed with DsbA, even though

its thiol group ionizes fully with a pK_a of about 3.5 (Nelson & Creighton, 1994).

Different results were observed with the reactivity of the Cys32 thiol group of Trx_{SH}^{SH} in thiol−disulfide exchange, which is a simple, well-understood reaction (Szajewski & Whitesides, 1980). This gave indications of only a single titration in Trx_{SH}^{SH} and Trx_{OH}^{SH},with an apparent pK_a of about 7 (Figures 3 and 5). But the reverse reaction with Trx_S^S, where GSH is the attacking thiol, showed the same apparent pH dependence, even though the pK_a of the GSH thiol group is well established to be about 8.8, nearly 2 pH units greater.

The kinetic date with thioredoxin are inconsistent and clearly do not reflect simply the ionization of the attacking thiol group. This inconsistency is perhaps not entirely surprising in this case, as thioredoxin is a catalyst of the reactions and therefore stabilizes the transition state for the reaction. The kinetic data may be reflecting primarily the ionization properties of the transition state, rather than of the protein itself.

The equilibrium data for the reaction with glutathione (Figure 3B) give a direct measure of the relative net charges of Trx_{SH}^{SH} and Trx_S^S. They clearly indicate that there is no net liberation or uptake of protons in the reaction Trx_{SH}^{SH} + GSSG ↔ Trx_S^S + 2GSH over the pH range 4−8, with only a slight liberation of protons at higher pH values. Other data are consistent with this conclusion: that Trx_{SH}^{SH} and Trx_S^S have the same net charge at about pH 8.6 had been demonstrated by Gleason (1992), and the equilibrium of Trx_{SH}^{SH} and Trx_S^S relative to NADP, catalyzed by thioredoxin reductase, had been shown to be independent of pH in the range 7−9 (Moore et al., 1964). If any other titrating groups of thioredoxin do have ionization properties different in Trx_{SH}^{SH} and Trx_S^S, their ionization between pH 4 and 9 must compensate that of the thiol groups of Trx_{SH}^{SH} and 2GSH exactly. It could be argued that the compensating group is Asp26, ionizing with pK_a > 9 in Trx_{SH}^{SH} but about 7.5 in Trx_S^S, as proposed by Wilson et al. (1995). Between pH 7.5 and 9.5, Trx_{SH}^{SH} would have Cys32 ionized, whereas Trx_S^S would have Asp26 ionized; the net charges of the two molecules would be the same. This explanation, however, does not explain why the thiolate anion in Trx_{SH}^{SH} was not observed at pH >7.5 by UV absorbance (Figure 6).

There is further, indirect evidence, from the effects on the stability of the folded protein of replacing each of the thiol groups, that the thiol groups of Trx_{SH}^{SH} have pK_a values in the region of 9. Replacing a cysteine residue that has a lower pK_a value in the folded state than in the unfolded should destabilize the protein, as was observed with DsbA (Zapun et al., 1994), and replacing a cysteine residue with a higher pH value in the folded state should have the opposite effect. Cysteine thiol groups in unfolded proteins generally have pK_a values close to 8.7. In thioredoxin, replacing Cys32 by Ser produced only small or negligible changes in the stability of the folded protein at pH 7 and 8.6 (Langsetmo et al., 1989), indicating that Cys32 has about the same pK_a value in the folded and unfolded states. Replacing Cys35 increased the stability of the native conformation (Langsetmo et al., 1989; Ladbury et al., 1994), suggesting that Cys35 normally has a higher pK_a value in the folded state, as would be expected (see Figure 6).

Furthermore, the equilibrium constant for simple thiol−disulfide exchange will depend upon the pK_a values of the two thiol groups, even if neither or both are ionized (Darby & Creighton, 1993). The equilibrium constant for the reaction Trx_{OH}^{SH} + GSSG ↔ Trx_{OH}^{SSG} + GSH had a value somewhat greater than 10 (Figure 5). This is consistent with the Trx_{OH}^{SH} thiol group having a substantially greater pK_a value than that of GSH, as was also indicated by the UV absorbance data (Figure 6). An equilibrium constant of 1 would be expected when the initial and starting thiol groups have the same pK_a value, unless, of course, there are other interactions between glutathione and thioredoxin in the mixed-disulfide Trx_{OH}^{SSG}.

The absorbance at 240 nm of the thiolate anion is a fundamental property of thiol groups (Benesch & Benesch, 1955), and it has given very satisfactory data with several proteins (Polgar, 1974; Graminski et al., 1989; LoBello et al., 1993; Nelson & Creighton, 1994; Grauschoph et al., 1995). In the present case, no indications of a substantial amount of thiolate anion in Trx_{SH}^{SH} or in the mutant proteins Trx_{OH}^{SH} and Trx_{SH}^{OH} were observed below pH 9. The expected absorbance appeared in each case only at about pH 10, although the exact pK_a values could not be determined accurately, due to the complications of tyrosine ionization and alkaline unfolding of the proteins. This is surely the most important indication of high pK_a values for the thiols of Trx_{SH}^{SH}.

There were some indications of small amounts of thiolate anion in Trx_{SH}^{SH} between pH 7 and 9, no more than 20% of one group; if significant, this observation may indicate the presence of a species between pH 7 and 9 where protons are largely on the cysteine thiols but shared to a significant extent with another group. One possibility is that a single proton is shared between the two thiol groups of Cys32 and Cys35, as proposed by Jeng et al. (1995), and that this somehow prevents the expected absorbance at 240 nm of the shared thiolate anion from being realized. The same effects would have been expected in DsbA, however, but ionization of a full thiol group was apparent at all pH values greater than 4 (Nelson & Creighton, 1994). Also, such an explanation is inconsistent with the observation that the net charges of Trx_{SH}^{SH} and Trx_S^S are the same below pH 9.

The simplest interpretation of all the data available on thioredoxin is that the two thiol groups of Trx_{SH}^{SH} have pK_a values in the region of 9−10 and that their ionization is not linked to any other groups to a substantial extent. Alternative, more complex interpretations cannot be ruled out completely, but each appears to have some inconsistencies with the experimental data. The simplest interpretation would also be consistent with the observations with DsbA and other members of this family (Grauschoph et al., 1995). It is often argued that a low pK_a value for Cys32 is important for the functional reactivity of Trx_{SH}^{SH}, as the thiolate anion is normally the reactive species. A low pK_a value does increase the population of the thiolate anion at low pH values, but this is offset by an intrinsically lower reactivity of that thiolate anion (Szajewski & Whitesides, 1980). The rate at pH 7 of reaction of a model thiol group is only 6 times greater with a pK_a of 7 rather than 9, even though there is a 50-fold difference in the concentration of thiolate anion. The pK_a value of the thiol group is more important for the stability of the disulfide bond, and a pK_a value of about 9 for Cys32

of Trx_{SH}^{SH} will account nicely for the measured stability of its disulfide bond (Holmgren, 1981; Grauschoph et al., 1995).

If the two thiol groups of Trx_{SH}^{SH} ionize fully only above pH 9, why were the thiolate anions of both identified by another direct method, Raman spectroscopy, and assigned apparent pK_a values of 7.1 and 7.9 (Li et al., 1993)? No explanation is certain, but it could conceivably be due to the very high protein concentrations (80−150 mg/mL) that were necessary for the Raman measurements. Trx_{SH}^{SH} dimerizes reversibly in a pH-dependent manner and more avidly than Trx_S^S (Holmgren, 1985; Ladbury et al., 1994). Therefore, high protein concentrations should stabilize Trx_{SH}^{SH} more than Trx_S^S, which will shift the equilibrium toward Trx_{SH}^{SH} and lower the apparent pK_a of its thiol groups. This would also explain why the buried Cys35 was observed by Raman spectroscopy also to ionize at such a relatively low pH, much lower than suggested by any other technique.

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