Reactivity and Ionization of the Active Site Cysteine Residues of DsbA, a Protein Required for Disulfide Bond Formation in Vivo

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Received January 18, 1994; Revised Manuscript Received March 7, 1994®

ABSTRACT: DsbA is a periplasmic protein of *Escherichia coli* that appears to be the immediate donor of disulfide bonds to proteins that are secreted. Its active site contains one accessible and one buried cysteine residue, Cys30 and Cys33, respectively, which can form a very unstable disulfide bond between them that is 10^3-fold more reactive toward thiol groups than normal. The two cysteine residues have normal properties when in a short peptide. In DsbA, the Cys30 thiol group is shown to be reactive toward alkylating reagents down to pH 4 and to be fully ionized, on the basis of the UV absorbance of the thiolate anion at 240 nm. Its reactivity is altered by another, unknown group on the reduced protein titrating with a pK_a of about 6.7. The other cysteine residue is buried and unreactive and has a high pK_a value. The ionization properties of the DsbA thiol groups can explain, at least partly, the high reactivity of its disulfide bonds and thiol groups at both neutral and acidic pH values.

Formation of disulfide bonds between cysteine residues is often an important step in the biosynthesis of proteins, as it frequently involves the folding, assembly, and stabilization of the native, biologically active structure. Not surprisingly, catalysts of protein disulfide bond formation are present at the appropriate locations where proteins are exported from the cytosol: protein disulfide isomerase (PDI)[1] within the endoplasmic reticulum of eukaryotes [reviewed by Freedman (1992)] and DsbA within the periplasm of Gram-negative bacteria (reviewed by Bardwell and Beckwith (1993)]. PDI is well-established to catalyze the intramolecular isomerization of protein disulfide bonds, which is often the rate-determining step in folding and formation of the correct disulfide bonds; it can also increase substantially the rate at which disulfide bonds are introduced into a protein (Creighton et al., 1980, 1993; Zapun et al., 1992). DsbA probably functions primarily by introducing disulfide bonds directly into the protein (Zapun et al., 1993; Wunderlich et al., 1993), but is much less effective than PDI at catalyzing intramolecular disulfide rearrangements (Zapun & Creighton, 1994). The mechanisms by which these catalysts function are being determined.

Both PDI and DsbA have structural and functional similarities to thioredoxin, a small protein of about 108 residues that functions primarily as a reductant [reviewed by Holmgren (1989)]. The active groups in thioredoxin are two cysteine thiol groups, occurring in the characteristic sequence -Cys-Gly-Pro-Cys-, that can be oxidized to form a disulfide bond between them. The disulfide bond of thioredoxin is then reduced by thioredoxin reductase, using NADPH of the cell. One of the cysteine thiol groups of thioredoxin projects from the three-dimensional structure (Katti et al., 1990; Dyson et al., 1990), consistent with its role in chemically attacking target disulfide bonds. The thioredoxin conformation also facilitates the formation of a stable disulfide bond between the two cysteine residues by keeping them in proximity.

The PDI polypeptide chain of about 500 residues contains two segments that are clearly homologous to thioredoxin; the two cysteine residues of each occur in the sequence -Cys-Gly-His-Cys- and are involved in the function of the protein (Freedman, 1992). These two cysteine residues are believed to form a disulfide bond reversibly, which is probably in equilibrium with the glutathione (GSH and GSSG) in the endoplasmic reticulum (Hwang et al., 1992). The structure of PDI is not known, but it is large and complex, containing domains other than those homologous to thioredoxin and having functions not directly related to protein disulfide bond formation (Freedman, 1992).

DsbA is considerably less complex, being a monomer of 189 residues with only two cysteine residues, in the sequence -Cys-Pro-His-Cys-. Although having no substantial sequence similarity, it is structurally homologous to thioredoxin, but with an insertion of an additional 76 amino acid residues (Martin et al., 1993). This insertion forms a helical subdomain adjacent to the -Cys-Pro-His-Cys- segment and converts the thioredoxin structure into one more typical of an enzyme, with two domains and a cleft, and the active site between them. The disulfide bond that forms reversibly in DsbA is, however, some 6.9 kcal/mol less stable than that of thioredoxin (Zapun et al., 1993; Wunderlich & Glockshuber, 1993). The corresponding disulfide bonds of PDI are also believed to be very unstable (Hawkins et al., 1991; Lyles & Gilbert, 1991; Lundström & Holmgren, 1993). The instability of the disulfide bond of DsbA makes it ideally suited to be the moiety that reacts with the cysteine residues of newly-synthesized proteins to join them with disulfide bonds when appropriate (Zapun et al., 1993), and the phenotypes of mutant bacterial strains lacking active DsbA are consistent with this being its

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® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

[1] Abbreviations: Cam, carbamoylmethyl group (the commonly used term for this group is "carboxamidomethyl"); Cm, carboxymethyl group; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; $DsbA_{SH}^{SH}$ and $DsbA_S^S$, the protein DsbA with free Cys30 and Cys33 thiol groups and with a disulfide bond between them, respectively; the upper sulfur atom is that of Cys30; the variants of DsbA with Cys30, Cys33, or both replaced by serine residues are designated as $DsbA_{SH}^{OH}$, $DsbA_{OH}^{SH}$, and $DsbA_{OH}^{OH}$, respectively, with the SH representing a cysteine residue, the OH a serine residue; DTT, dithiothreitol; ϵ_x, extinction coefficient at x nm; EDTA, ethylenediaminetetraacetic acid; GSH and GSSG, the reduced and oxidized forms of glutathione, respectively; HPLC, high-pressure liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PDI, protein disulfide isomerase; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/94/0433-5974$04.50/0

primary physiological role (Bardwell et al., 1991). The unstable-disulfide bond of DsbA is believed to be regenerated by an integral membrane protein, DsbB (Bardwell et al., 1993).

DsbA is a relatively simple system with which to elucidate the mechanism of action of catalysts of protein disulfide bond formation. The first goal is to understand the energetics of its unstable intramolecular disulfide bond and the even more unstable mixed disulfide it forms with glutathione (Zapun et al., 1993, 1994). Even though the disulfide bonds of DsbA and of thioredoxin differ by 6.9 kcal/mol in free energy, they are structurally superimposable (Martin et al., 1993). The second goal is to understand the rapid rates at which DsbA undergoes thiol–disulfide exchange with other thiols and disulfides (Zapun et al., 1993, 1994). Finally, DsbA is remarkably effective at putting disulfide bonds into proteins at acid pH, as low as pH 4 (Wunderlich et al., 1993; Zapun & Creighton, 1994). Thiol–disulfide exchange is normally very slow at acid pH, because the ionized thiolate anion is the reactive species, and a typical cysteine thiol group has a pK_a of about 8.7. Yet this reaction is very rapid with DsbA. This is probably especially important physiologically, to permit bacteria such as *Escherichia coli* to grow at pH values as low as 4.4 (Wunderlich et al., 1993).

A fundamental aspect of protein thiol groups is their pK_a values. This determines the extent of their ionization at any particular pH value and, consequently, their reactivity in thiol–disulfide exchange reactions. But the pK_a value of the thiol group also reflects the intrinsic chemical reactivity of the sulfur atom, including its reactivity when it is part of a disulfide bond (Creighton, 1975). The rate of thiol–disulfide exchange between an ionized thiol group and each of the two sulfur atoms of a disulfide bond can be predicted accurately in model systems if the pK_a values are known for the three sulfur atoms when in the thiol form (Szajewski & Whitesides, 1980; Houk et al., 1987). For that reason, we have studied the ionization properties of the two thiol groups of DsbA. They have markedly different properties, and one is fully ionized at acidic pH values. The results provide explanations for some of the functional properties of DsbA, and probably also for other members of the thioredoxin family.

EXPERIMENTAL PROCEDURES

Chemicals. GSH, GSSG (grade III), and iodoacetamide were obtained from Sigma Chemical Co. DTT was from Saxon Biochemicals GmbH (Hannover, Germany). TFA was Sigma sequencing grade. All other buffers and chemicals were of the highest purity commonly available.

Proteins. The various forms of DsbA were prepared as described previously (Zapun et al., 1993, 1994). The same ϵ_{280} of 21 740 M^{-1} cm^{-1} was used for all the proteins (Zapun et al., 1993, 1994). $DsbA_{SH}^{SH}$ and $DsbA_{S}^{S}$ were prepared by adding either 10–50 mM DTT or 5 mM GSSG, respectively, to the protein in 0.1 M Tris·HCl, pH 8.5, before loading on a 1 cm × 20 cm column of Sephadex G-25 equilibrated and run in the appropriate buffer.

The peptide acetyl-Phe-Cys-Pro-His-Cys-Tyr-amide was synthesized using 9-fluorenylmethoxycarbonyl chemistry and purified to >95% homogeneity by reverse phase HPLC. The reduced form contained 1.87 thiols as determined by the method of Ellman (1959). The expected ϵ_{280} of the reduced peptide was measured by the method of Gill and von Hippel (1989) to be 1200 M^{-1} cm^{-1}.

Buffers. All buffer solutions contained 0.2 M KCl and 1 mM EDTA. The reaction of ICam with the proteins and peptides was measured at pH > 4 in a buffer consisting of 10 mM each of acetate (pK_a = 4.76), MES (pK_a = 6.1), MOPS (pK_a = 7.2), and Tris (pK_a = 8.1), adjusted to the appropriate pH with KOH or HCl. With the peptide, the buffer also contained 10 mM NH_3 (pK_a = 9.24). For pH < 4, the buffers contained 10 mM each of acetate, glycylglycine (pK_a = 3.12), and glycine (pK_a = 2.34). The absorbance measurements were made in a buffer containing 1 mM each of phosphate (pK_a = 2.15, 7.20, and 12.38), citrate (pK_a = 3.13, 4.76, and 6.40), and borate (pK_a = 9.24). The kinetics of reaction between DsbA and glutathione at pH 4 were measured in 50 mM acetic acid adjusted to pH 4 with KOH. The equilibrium between DsbA and glutathione was measured in individual 50 mM buffers of barbiturate, formate, acetate, citrate, MES, MOPS, Tris, ammonia, CHES, or borate. Each of these buffers gave results that were very similar to those from at least one of the other buffers at the same pH. The pH values were measured using either a standard (GK2421C) or micro (GK2401C) Radiometer pH electrode with a Radiometer PHM 83 pH meter.

The temperatures of the solutions were maintained at 25 °C by thermostated sample holders using a circulating water bath for the spectral measurements. Other reactions were performed in vials immersed in a 25 °C water bath. Important volumes for kinetic experiments were measured using Hamilton syringes, always using at least 40% of the syringe capacity.

Glutathione Concentrations. GSH concentrations were measured by the method of Ellman (1959) as described by Creighton (1989); an ϵ_{412} of 14 140 M^{-1} cm^{-1} (Riddles et al., 1983) was used for the thionitrobenzoate anion. GSSG concentrations were measured by the ϵ_{248} of 381 M^{-1} cm^{-1} (Chau & Nelson, 1992).

Kinetics of Reaction with ICam. For $DsbA_{SH}^{SH}$ and $DsbA_{OH}^{SH}$, 10 μL of 100 mM ICam was added to 1.0 mL of a 25–30 μM solution of protein or peptide; the solution was thoroughly mixed, and the change in absorbance at 267 nm was measured in a 1.0-cm-path-length cuvette in a Uvikon 930 UV–vis absorbance spectrometer (Kontron). The rate of the reaction was measured at pH values between pH 3.5 and 9 for the protein and between pH 6 and 10 for the peptide; for the latter at pH 6, where the reaction was very slow, a concentration of 10 mM ICam and a 1-mm-path-length cuvette were used. The change in absorbance (Abs) with time (t) was fitted to an exponential: $Abs(t) = Abs(\infty) + A \exp(-t/\tau)$, where τ is the relaxation time for the reaction, using the nonlinear least-squares routine in KaleidaGraph (Abelbeck Software). The nonlinear least-squares routine was also used to fit the resulting plot of $k_{obs} = 1/(\tau[ICam])$ vs pH to eq 1 for the peptide and eq 2 for the proteins (see below). The errors in the values of τ, and hence for the rate constants, are estimated to be on the order of 15%, based on repeated measurements at the same pH; errors in the pK_a values are estimated to be approximately 0.2 pH units. The errors in the amplitudes of the absorbance change are estimated to be approximately 20% for the proteins and 10% for the peptide, which had double the concentration of reactive thiol. Most of this error is a result of uncertainties in extrapolating back to time 0, due to the time required for manual mixing.

UV Absorbance Spectra. Absorbance spectra at pH 5.0 were measured for all proteins at 5–10 μM concentrations in 1.0-cm-path-length cuvettes. Spectra were taken in 0.5-nm steps from 370 to 240 nm, using the Kontron spectrophotometer. The protein solution was scanned vs air, followed by the buffer solution in the same cuvette vs air; the two spectra were then subtracted and the difference converted to molar extinction coefficients.

For spectra taken as a function of pH, 2.0 mL of the protein solution was added to a 1.0-cm-path-length, 3.0-mL-volume stoppered cuvette, with a small stir bar for mixing of the solution. After the pH of the solution was adjusted, the spectrum was recorded vs air. The pH was then adjusted by adding measured volumes of 0.02 M KOH, 0.02 M HCl, or 0.1 M HCl, prepared in the same buffer solution. A matched cuvette contained only buffer and was manipulated in the same manner. Separate spectra were recorded of the protein sample and buffer, as above. The buffer spectrum was subtracted from the protein spectrum, a correction was made for small differences in absorbance by the cuvettes and for the dilution caused by pH adjustment, and the data were converted to molar extinction coefficients. Separate samples were run from pH 5.0 to 10.0 and from pH 5.0 to 2.0.

Reaction between DsbA and Glutathione. The mechanism and rate of the thiol–disulfide exchange reaction between DsbA and glutathione at pH 4 (Wunderlich et al., 1993) were investigated by reverse phase HPLC to measure the species trapped during the course of the reaction (Zapun et al., 1993, 1994). The reaction was initiated by mixing equal volumes of 9–10 μM $DsbA^{SH}_{SH}$ or $DsbA^{S}_{S}$ and of GSH or GSSG at various concentrations. The thiol–disulfide exchange reaction between them was quenched at various times by adding 25 μL of 0.1 M HCl to 25 μL of reaction mixture, reducing the pH to below 2. The equilibrium between DsbA and glutathione was measured by adding $DsbA^{S}_{S}$ to appropriate mixtures of excess GSH and GSSG and permitting them to come to equilibrium for a few minutes, before trapping the species with an equal volume of 0.1 M HCl. Separations of the trapped species were performed on a Vydac 218TP54 C_{18} reverse phase column heated to 25 or 38 °C on a Gilson Model 305 HPLC apparatus. Elution was with linear gradients of acetonitrile in 0.1% (v/v) TFA. The absorbance was monitored simultaneously at 220 and 280 nm with a Gilson Model 116 HPLC absorbance monitor. Integrations of peak areas were performed with the Gilson HPLC software. The $DsbA^{SH}_{SH}$, $DsbA^{S}_{S}$, and $DsbA^{SSG}_{SH}$ species are well-separated under these conditions (Zapun et al., 1993, 1994).

The reaction between DsbA and glutathione was also monitored by the change in fluorescence of DsbA (Zapun et al., 1993; Wunderlich & Glockshuber, 1993). Fluorescence intensities were measured as a function of time by using either an SLM Aminco 8000 or Aminco Bowman Series 2 fluorescence spectrophotometer. A 4-mm square cuvette was used, with excitation at 280 nm and a 1-nm bandwidth; emission was monitored at 320 nm with an 8-nm bandwidth.

RESULTS

When the states of the cysteine residues of DsbA are specified, the sulfur atom of Cys30 is depicted as the superscript, $DsbA^{S}$, that of Cys33 as the subscript, $DsbA_{S}$. Thiol groups are depicted as "SH", irrespective of their degree of ionization. When a cysteine residue has been replaced by serine, it is depicted as "OH".

pH Dependence of the Rate of Reaction with Iodoacetamide. The reaction of cysteine thiol groups with a reagent like iodoacetamide (ICam), to form the Cam-Cys residue, is well-characterized and established to occur only with the ionized thiolate anion (Jocelyn, 1972). Consequently, measuring the rate of reaction as a function of pH can be used to measure the pK_a values of cysteine thiol groups (Creighton, 1975; Polgár & Halász, 1978; Snyder, 1987). The observed rate of the reaction, k_{obs}, should be proportional to the fraction of thiolate anion, f_{s^-}, according to the equation

$$k_{obs} = k_{s^-} f_{s^-} = \frac{k_{s^-}}{1 + 10^{pK_a - pH}} \quad (1)$$

where k_{s^-} is the rate constant for reaction of the thiolate anion.

The technique employed here takes advantage of the UV absorbance of ICam, with ϵ_{265} of 390 M^{-1} cm^{-1} (Polgár, 1974), and of the thiolate anion, with ϵ_{240} of about 4000 M^{-1} cm^{-1} (Benesch & Benesch, 1955; Polgár, 1974); both disappear upon reaction between the two. As a test of the method, the kinetics of reaction were measured with a peptide containing two cysteine residues and corresponding to the active site of DsbA, acetyl-Phe-Cys-Pro-His-Cys-Tyr-amide. The reaction was monophasic, and the observed amplitude of the spectral change was about that expected for two thiol groups (Figure 1b). The rate varied with the pH in the expected manner (Figure 1a), with a k_{s^-} value of 25 s^{-1} M^{-1} and the rate decreasing to 0 at low pH values, with an apparent pK_a of 8.7 (Table 1). The pK_a values of the two cysteine thiol groups must be very similar, and the observed value would be the average of the two. Very similar values of k_{s^-} = 20 s^{-1} M^{-1} and pK_a = 8.8 were measured previously for the Cys14 and Cys38 thiol groups of partly-reduced bovine pancreatic trypsin inhibitor (Creighton, 1975), and similar values of pK_a and reactivity have been measured with other unstructured peptides (Snyder, 1987). At moderate ionic strengths like those used here, the pK_a values of the cysteine thiol groups of unstructured peptides have been shown to depend only slightly upon the amino acid sequence, being affected somewhat by charged groups on adjacent residues (Snyder et al., 1981). Therefore, the similarities in the results obtained with different cysteine residues and peptides are to be expected.

Very atypical results were obtained with both $DsbA^{SH}_{SH}$ and $DsbA^{SH}_{OH}$ (Figure 1). Under these conditions, only Cys30 of $DsbA^{SH}_{SH}$ reacts at a substantial rate (Zapun et al., 1993, 1994). The Cys30 thiol group of both proteins exhibited high reactivity at pH values greater than 8, with rate constants of 79 and 37 s^{-1} M^{-1}, respectively. The rate decreased between pH 8 and 6, but did not become insignificant. Instead, the rate reached plateau values of 18 and 8 s^{-1} M^{-1} for $DsbA^{SH}_{SH}$ and $DsbA^{SH}_{OH}$, respectively. These values persisted down to pH 4. The rates of reaction of Cys30 observed at low pH are typical of those of fully ionized thiols, normally observed only at high pH, whereas the rates with Cys30 at high pH are substantially greater.

The rate of reaction of $DsbA^{SH}_{SH}$ was uniformly twice that of $DsbA^{SH}_{OH}$, but this was not a result of Cys33 reacting in $DsbA^{SH}_{SH}$. Instead, replacing Cys33 by Ser decreased the reactivity of the Cys30 thiol with ICam; a similar effect has been observed in its thiol–disulfide exchange reaction with glutathione (Zapun et al., 1994). Cys33 apparently has a general effect on the reactivity of Cys30, as has also been observed with the related glutaredoxin (Yang & Wells, 1991).

The pH dependence of the rate of reaction of both $DsbA^{SH}_{SH}$ and $DsbA^{SH}_{OH}$ followed that expected for titration of a single group, but with plateau rates at both high and low pH values, k_{hi} and k_{lo}, respectively:

$$k_{obs} = k_{lo} + \frac{k_{hi} - k_{lo}}{1 + 10^{pK_a - pH}} \quad (2)$$

The apparent pK_a values observed for $DsbA^{SH}_{SH}$ and $DsbA^{SH}_{OH}$ were 6.8 and 6.6, respectively (Table 1). This behavior could have two explanations: either (1) the Cys30 thiol group of each protein had a pK_a value near 6.7 and was reactive in the nonionized, thiol form, which can occur in

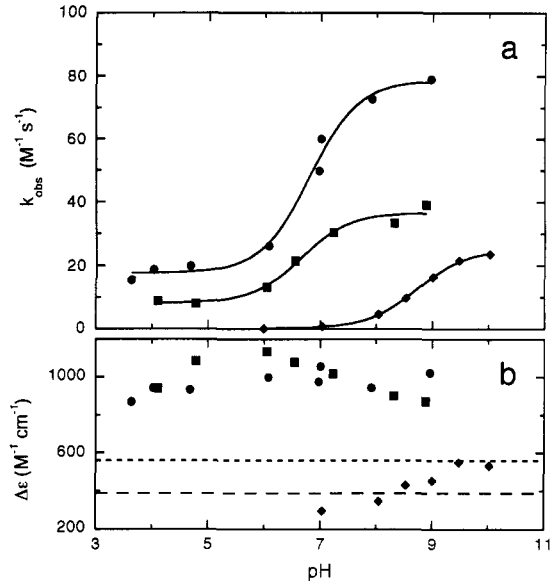


FIGURE 1: The kinetics of reaction of the thiol groups of $DsbA_{SH}^{SH}$ (●), $DsbA_{OH}^{SH}$ (■), and the peptide acetyl-Phe-Cys-Pro-His-Cys-Tyr-amide (◆) with iodoacetamide as a function of pH. The apparent second-order rate constants are given in part a; the amplitudes of the spectral change in the reaction are given in part b. Also indicated are the absorption changes expected for the reaction of iodoacetamide with a nonionized thiol group (– – –) and with an ionized thiolate anion (- - -).

Table 1: Effect of pH on the Rate of Reaction of Cys Thiol Groups with Icam[a]

thiol	k_{lo} (s^{-1} M^{-1})	k_{hi} (s^{-1} M^{-1})	pK_a
peptide[b]	0	25 ± 1	8.72 ± 0.02
$DsbA_{SH}^{SH}$	18 ± 2	79 ± 5	6.80 ± 0.09
$DsbA_{OH}^{SH}$	8 ± 1	37 ± 3	6.65 ± 0.12

[a] The experimental data were fitted to eq 1 for the peptide and eq 2 for Dsb_{SH}^{SH} and $DsbA_{OH}^{SH}$ with the nonlinear least-squares routine in KaleidaGraph; the error estimates are those provided by the program. [b] Acetyl-Phe-Cys-Pro-His-Cys-Tyr-amide.

special circumstances (Grimshaw et al., 1979; Roberts et al., 1986), or (2) the thiol group remained ionized at all pH values, and the change in reactivity was due to the titration of another group.

The measured amplitudes of the absorbance change during the reaction gave some clues. The amplitude is expected at high pH values to be the sum of that due to the absorbance of ICam and of the thiolate anion, about 560 M^{-1} cm^{-1}, whereas at low pH, below the pK_a of the thiol, it should be due to just the ICam. The amplitudes observed with the peptide are close to those expected, and they changed with pH in the expected manner (Figure 1b). In contrast, the amplitudes observed with the proteins were considerably greater in value and relatively constant with pH. This suggests that the thiolate anion is present at all pH values, but that there are probably additional changes in the absorbance of the protein upon reaction with ICam.

Measurement of the Ionization of Thiol Groups by UV Absorbance. The thiolate anion can be detected readily by its absorbance at around 240 nm (Benesch & Benesch, 1955; Polgár, 1974; Graminski et al., 1989; Lo Bello et al., 1993). Because many other groups in a protein also absorb in this wavelength region, the absorbance of the thiolate anion must be detected by comparison with the spectrum of the same protein in which the thiol group is absent. Previous studies have used the protein in which the thiol has been reacted with ICam (Polgár, 1974), but it is now practical to use protein

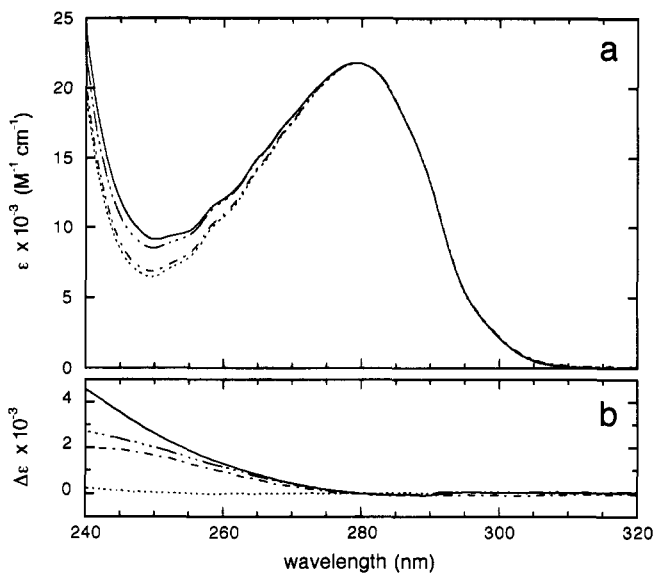


FIGURE 2: Absorbance properties at pH 5.0 of the various forms of DsbA. (a) Absorbance spectra of $DsbA_{SH}^{SH}$ (—), $DsbA_{OH}^{SH}$ (– ··· –), $DsbA_{OH}^{OH}$ (···), and $DsbA_{OH}^{SCam}$ (– · –). The spectrum of $DsbA_{SH}^{OH}$ was identical to that of $DsbA_{OH}^{OH}$. (b) Difference spectra: $DsbA_{SH}^{SH}$ minus $DsbA_{OH}^{OH}$ (—), $DsbA_{OH}^{SH}$ minus $DsbA_{OH}^{OH}$ (– ··· –), $DsbA_{OH}^{SH}$ minus $DsbA_{OH}^{SCam}$ (– · –), and $DsbA_{SH}^{OH}$ minus $DsbA_{OH}^{OH}$ (···).

engineering to replace Cys residues, as in the variants of DsbA: $DsbA_{SH}^{OH}$, $DsbA_{OH}^{SH}$, and $DsbA_{OH}^{OH}$ (Zapun et al., 1994). The UV absorbance spectra can also be compared to that of $DsbA_{S}^{S}$, where there is a disulfide bond between the two thiol groups. The very similar spectral and physical properties of these engineered variants of DsbA, including the chemically modified forms $DsbA_{SH}^{SCam}$ and $DsbA_{OH}^{SCam}$, indicate that all have similar structures (Zapun et al., 1993, 1994).

The absorbance spectra of these forms of DsbA at pH 5 are shown in Figure 2. All the spectra were essentially identical at wavelengths greater than 270 nm, as expected. Between 240 and 270 nm, the spectra fall into two classes: (1) $DsbA_{S}^{S}$, $DsbA_{SH}^{OH}$, $DsbA_{OH}^{SCam}$, and $DsbA_{OH}^{OH}$, which exhibit spectra that essentially overlap, with low absorbance in the region of the minimum at 250 nm; none of these proteins have a Cys30 thiol group; (2) $DsbA_{SH}^{SH}$ and $DsbA_{OH}^{SH}$, which have greater absorbance in this region; both have the Cys30 thiol group. These spectra indicate that the Cys30 thiol is substantially ionized at pH 5.

To investigate further, the absorbance spectra of $DsbA_{SH}^{SH}$ and $DsbA_{OH}^{OH}$ were compared between pH 2 and 10 (Figure 3). The spectra of both proteins changed most dramatically at extremes of pH. At high pH values, the absorbance increased below 260 nm and above 290 nm, presumably due to ionization of tyrosine residues. At pH 2, the spectra of both proteins were altered over the entire wavelength region, probably due to unfolding of the protein and exposure of the buried tyrosine residues (R. Glockshuber, personal communication). At intermediate pH values, both spectra changed relatively little with pH. The spectrum of $DsbA_{OH}^{OH}$ was virtually the same at pH 3 and 5, whereas the absorbance of $DsbA_{SH}^{SH}$ increased at wavelengths below 260 nm at pH 5. This increase in absorbance below 260 nm is indicative of the ionization of thiol groups.

The values of ε at 240, 288, and 295 nm as a function of pH are shown in Figure 4. Changes at 240 nm reflect ionization of thiols; at 288 nm, unfolding; and at 295 nm, tyrosine ionization. The two proteins $DsbA_{SH}^{SH}$ and

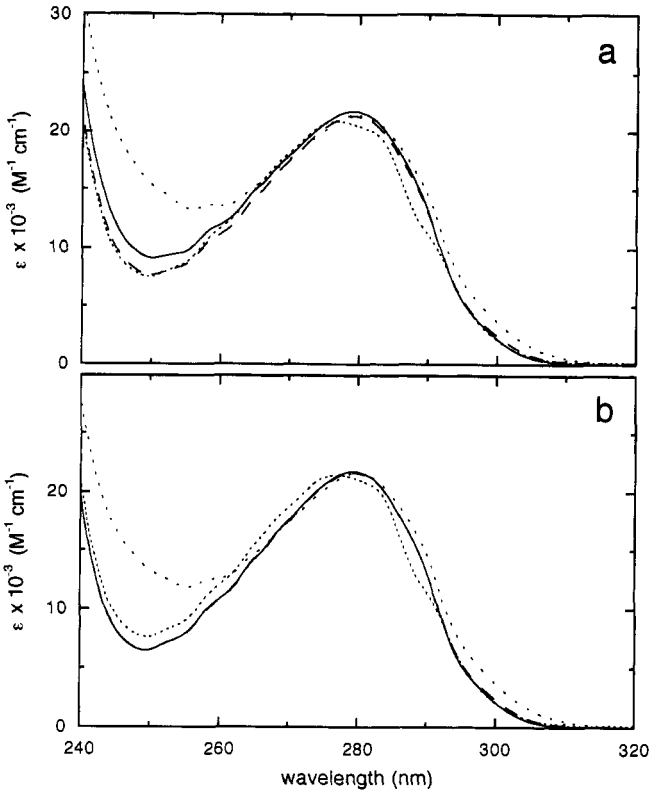


FIGURE 3: Absorbance spectra of $DsbA^{SH}_{SH}$ (a) and $DsbA^{OH}_{OH}$ (b) at pH 2.0 (···), 3.1 (– – –), 5.0 (—) and 10.0 (- - -). In part b, the spectra at pH 3.1 and 5.0 overlap.

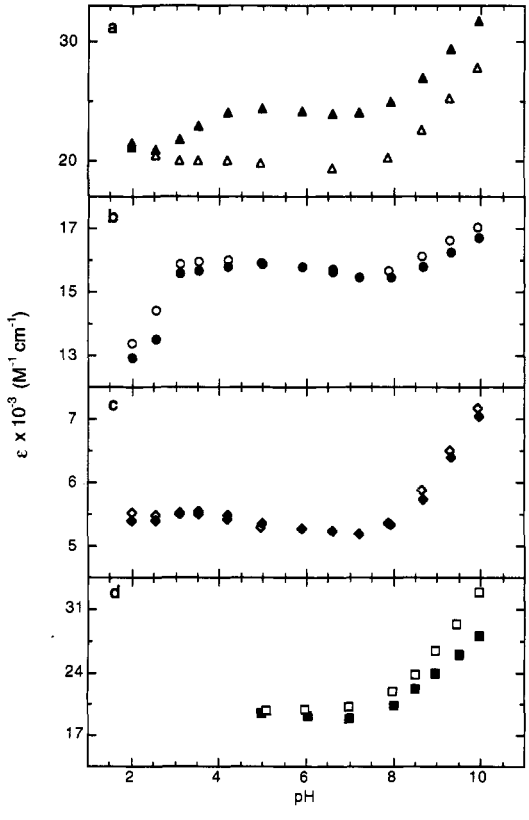


FIGURE 4: Ionization of the Cys30 and Cys33 thiol groups. (a–c) Absorbance properties of $DsbA^{SH}_{SH}$ (solid symbols) and of $DsbA^{OH}_{OH}$ (open symbols) as a function of pH. The wavelengths were (a) 240 nm, (b) 288 nm, and (c) 295 nm. (d) Absorbance properties of $DsbA^{OH}_{SH}$ (□) and $DsbA^{OH}_{OH}$ (■) at 240 nm as a function of pH.

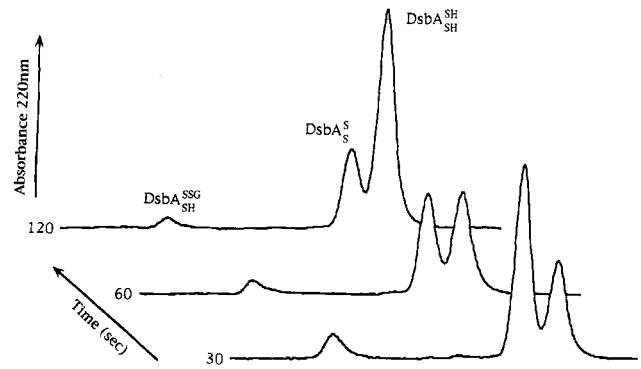
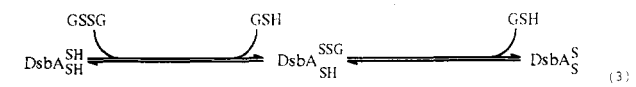


FIGURE 5: Reverse-phase HPLC profiles of the species of DsbA trapped by acid during reaction with 3 mM GSH at pH 4.0. The initial starting material contained only $DsbA^{S}_{S}$. Upon adding GSH, a small quantity of the mixed disulfide $DsbA^{SSG}_{SH}$ was generated very rapidly, followed by gradual conversion to $DsbA^{SH}_{SH}$. The other mixed disulfide, $DsbA^{SH}_{SSG}$, can be generated with unfolded DsbA in 8 M urea and was shown to elute nearly midway between $DsbA^{SSG}_{SH}$ and $DsbA^{S}_{S}$; it is not detected with folded DsbA.

$DsbA^{OH}_{OH}$ exhibited almost identical values of ϵ and pH dependence at 288 and 300 nm, indicating that they had very similar behavior with respect to tyrosine ionization above pH 8 and acid unfolding below pH 3. Circular dichroism spectra confirmed that $DsbA^{SH}_{SH}$ was fully folded above pH 3 (data not shown). Very different spectral properties were exhibited at 240 nm, however; there was a significant and constant difference in ϵ at pH values greater than 4. This difference is that expected if the Cys30 thiol group of $DsbA^{SH}_{SH}$ is fully ionized above pH 4 and has a pK_a value of about 3.5. Protonation is complete only at pH 2.5, where both proteins appear to be denatured.

All of the spectral measurements indicate that Cys30 of DsbA is ionized at all pH values greater than 4. In contrast, Cys33 appears to be protonated in $DsbA^{SH}_{SH}$ over the entire pH range, up to pH 10. This is indicated by the absence of a second ionization at high pH for $DsbA^{SH}_{SH}$ when compared to $DsbA^{OH}_{OH}$ (Figure 4a). In the absence of Cys30, however, as in $DsbA^{OH}_{SH}$, Cys33 was observed by the increase in ϵ_{240} to ionize with a pK_a value estimated to be approximately 9.5 (Figure 4d). The electrophoretic mobility of $DsbA^{OH}_{SH}$ also indicated that Cys33 was about half ionized at pH 9.5 (Zapun et al., 1994). The ionization of Cys33 is probably inhibited by the presence of ionized Cys30 in $DsbA^{SH}_{SH}$.

Thio-Disulfide Exchange of DsbA with Glutathione at Acidic pH. The low pK_a value of the Cys30 thiol group helps to explain the activity of DsbA at acidic pH (Wunderlich et al., 1993). It therefore was pertinent to examine the reaction between DsbA and glutathione at acidic pH and to compare it to that at pH 7.5 (Zapun et al., 1993, 1994). The reaction proceeds through an unstable mixed disulfide on Cys30:



The reaction can be monitored by trapping all the species at low pH and separating and quantifying the DsbA species by reverse phase HPLC or by using the fluorescence change that accompanies interconversion of $DsbA^{SH}_{SH}$ and $DsbA^{S}_{S}$ (Zapun et al., 1993, 1994; Wunderlich & Glockshuber, 1993).

The reaction was monitored in both directions, starting with either $DsbA^{SH}_{SH}$ or $DsbA^{S}_{S}$. Figure 5 presents HPLC profiles of aliquots trapped during reduction of $DsbA^{S}_{S}$ by GSH. In

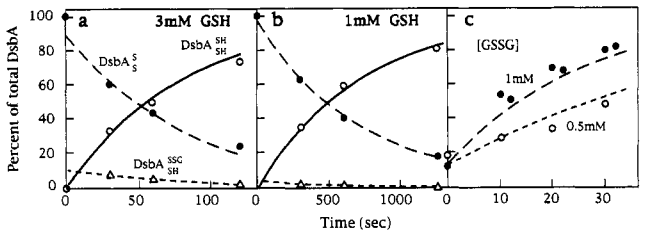


FIGURE 6: Kinetics of reaction of DsbA with glutathione at pH 4. The reaction was measured by reverse phase HPLC separation of acid-trapped species, as in Figure 5. (a, b) Kinetics of reaction between 5 μM $DsbA_S^S$ and either (a) 3.0 mM or (b) 1.0 mM GSH. The fractions of DsbA molecules present as (●) $DsbA_S^S$, (△) $DsbA_{SH}^{SSG}$, and (○) $DsbA_{SH}^{SH}$ are plotted as a function of time. The curves are the theoretical results expected if $DsbA_S^S$ and GSH were in rapid equilibrium with $DsbA_{SH}^{SSG}$, with an equilibrium constant of 0.025 M, and where the overall rate constant for the reaction was 1.4×10^3 s^{-1} M^{-2}. (c) Kinetics of reaction between 5 μM $DsbA_{SH}^{SH}$ and either 0.5 mM (○) or 1.0 mM (●) GSSG. The fraction of molecules present as $DsbA_S^S$ is plotted as a function of time; the starting material contained about 14% $DsbA_S^S$. The curves are the results expected with a rate constant of 40 s^{-1} M^{-1}.

this direction, the mixed-disulfide species $DsbA_{SH}^{SSG}$ accumulated to significant levels. It was generated very rapidly upon mixing $DsbA_S^S$ with GSH, to a level proportional to the concentration of GSH, and it disappeared at about the same rate as $DsbA_S^S$ (Figure 6a). These observations indicate that the interconversion between $DsbA_S^S$ and $DsbA_{SH}^{SSG}$ is relatively rapid and that they were nearly in equilibrium during the reaction, with the equilibrium constant K_2 having the value 0.025 ± 0.003 M. The individual rate constants that make up

$$K_2 = \frac{[DsbA_S^S][GSH]}{[DsbA_{SH}^{SSG}]} \quad (4)$$

this equilibrium are not determined by these data. The overall rate of appearance of $DsbA_{SH}^{SH}$ upon reduction of $DsbA_S^S$ was demonstrated to be second order in the concentration of GSH, as expected, with an overall third-order rate constant of $(1.4 \pm 0.1) \times 10^3$ s^{-1} M^{-2}.

The reaction between $DsbA_{SH}^{SH}$ and GSSG was first order in the concentration of GSSG, and no $DsbA_{SH}^{SSG}$ intermediate accumulated (Figure 6b). In this case, the overall rate of the reaction is determined by the first step, the formation of $DsbA_{SH}^{SSG}$, and gives the second-order rate constant, 40 s^{-1} M^{-1}. That no $DsbA_{SH}^{SSG}$ intermediate accumulated with half-times of reaction as short as 10 s indicates that the rate constant for its intramolecular conversion to $DsbA_S^S$ must be greater than 1 s^{-1}.

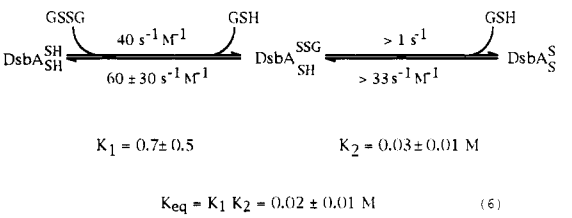
The overall rates of the reaction in the two directions indicate that the overall equilibrium constant for the reaction, K_{eq}, should have the value 29 mM. Equilibrium mixtures analyzed

$$K_{eq} = \frac{[DsbA_S^S][GSH]^2}{[DsbA_{SH}^{SH}][GSSG]} \quad (5)$$

directly gave the value 10 mM. The intermediate $DsbA_{SH}^{SSG}$ accumulated to the expected levels and gave essentially the same value of K_2, 0.03 M, as obtained from the kinetic experiments. Very similar values of both equilibrium constants were measured with 50 mM acetate, formate, or barbiturate buffers at pH 4.

The slight discrepancy in the kinetic and equilibrium data may be due to inadequacies of the acid-trapping reaction. The unusually rapid rates of the thiol–disulfide exchange reaction between DsbA and glutathione at pH 4 probably make it difficult to quench the reaction by lowering the pH. The pH must be lowered to less than 3 before Cys30 is protonated, and unfolding of DsbA by acid may be necessary before the reaction is adequately slowed. The conversion of $DsbA_{SH}^{SSG}$ to $DsbA_S^S$ is rapid and intramolecular and would be expected to be especially difficult to quench; indeed, the amount of $DsbA_{SH}^{SSG}$ trapped was found to vary with the buffers used and with the method of acidification. The results presented here were obtained with a method that produced the greatest amount of $DsbA_{SH}^{SSG}$, and it seems likely, but is not certain, that the quenching reaction was adequate. Much less variation was observed in the relative amounts of $DsbA_{SH}^{SH}$ and $DsbA_S^S$, as would be expected, and the fidelity of this trapping reaction was illustrated by the general pseudo-first-order kinetics of reaction measured in both directions by acid trapping (Figure 6). Also, very similar rate constants were measured directly using the fluorescence difference between $DsbA_{SH}^{SH}$ and $DsbA_S^S$. Nevertheless, small differences in the amounts of $DsbA_{SH}^{SH}$ and $DsbA_S^S$ trapped could produce small discrepancies between the kinetic and equilibrium experiments.

Consideration of all the data indicates that the reaction between DsbA and glutathione at pH 4 is described by the kinetic scheme



pH Dependence of the Equilibrium between DsbA and Glutathione. If the Cys30 thiol group of $DsbA_{SH}^{SH}$ has a pK_a value of 3.5, while that of GSH is approximately 8.8, the values of the overall equilibrium constant, K_{eq} of eq 6, and of K_1 should vary with pH in the range 4–8. In general, a plot of log K_{eq} *versus* pH should have a slope equal to the net number of protons liberated in the reaction. GSH and GSSG do not differ substantially in their ionization properties other than the thiol group (Jung et al., 1972), so the variation in equilibrium constant at pH values between 4 and 8 should reflect primarily the differences in ionization of $DsbA_{SH}^{SH}$, $DsbA_{SH}^{SSG}$, and $DsbA_S^S$. Consequently, if only Cys30 has an unusual pK_a value and no other ionizations in DsbA are linked to disulfide bond formation, the value of the equilibrium constant between them, K_{eq}, should be 10^4 times greater at pH 4 than at pH 8. The pH dependence resulting from the low pK_a value of Cys30 should be apparent in the equilibrium between $DsbA_{SH}^{SH}$ and $DsbA_{SH}^{SSG}$, given by the equilibrium constant K_1. The second step between $DsbA_{SH}^{SSG}$ and $DsbA_S^S$ should be independent of pH in this range, but should vary at higher pH values in the region where the Cys33 and GSH thiol groups ionize differently.

The equilibrium between DsbA and glutathione was measured at pH values between 4 and 9, using acid-trapped samples as previously (Figure 7). The concentrations of GSH and GSSG were in the range 1–20 mM, far exceeding the concentration of DsbA (about 5 μM); they were varied to produce nearly equal amounts of $DsbA_{SH}^{SH}$ and $DsbA_S^S$ and to give an accurate value of the overall equilibrium constant. At least two different buffers were used at each pH and shown

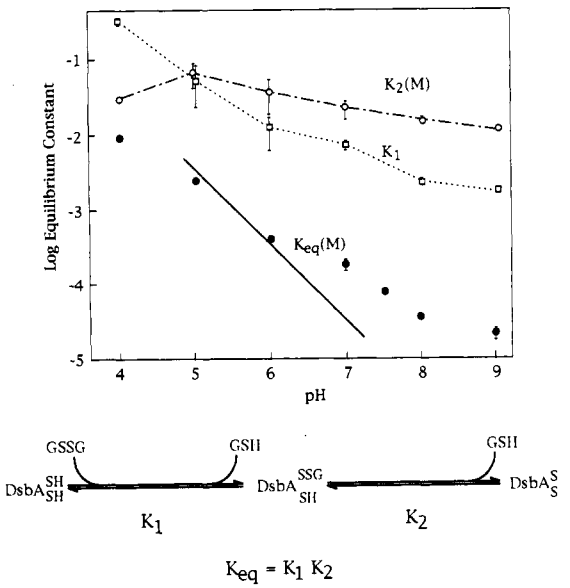


FIGURE 7: pH dependence of the equilibrium constants for the reaction between DsbA and glutathione. The fractions of DsbA molecules present at equilibrium with various excess concentrations of GSH and GSSG as the three different forms were determined by reverse-phase HPLC analysis of acid-quenched samples, as in Figure 5, to determine the values of the partial equilibrium constants K_1 (□) and K_2 (○) and the overall equilibrium constant K_{eq} (●). The values of K_2 and K_{eq} have units of molarity. The mean deviations of multiple measurements are indicated; where none is visible, the mean deviation was within the dimensions of the symbol. The solid line has the slope −1, which is the expected pH dependence if a net of one proton is taken up by the reaction.

to give very similar results, to ensure that the buffer was not altering the results. Qualitatively similar data have been reported by Wunderlich et al. (1993), measured using the fluorescence difference between $DsbA_{SH}^{SH}$ and $DsbA_{S}^{S}$, but the data diverge quantitatively at acidic pH. The difference may be due in part to the inability of fluorescence measurements to distinguish the mixed disulfide, which accumulates to greater extents at low pH.

In each case, the accumulation of the mixed disulfide, $DsbA_{SH}^{SSG}$, permitted measurement of both half equilibrium constants, K_1 and K_2. The levels of the mixed disulfide were more variable than those of $DsbA_{SH}^{SH}$ and $DsbA_{S}^{S}$, differing somewhat with different methods of acid trapping and also with different buffers. It is likely that the intramolecular nature of the conversion of $DsbA_{SH}^{SSG}$ to $DsbA_{S}^{S}$ and its rapid rate at acidic pH make it difficult to trap $DsbA_{SH}^{SSG}$ by acidification, as discussed above. Consequently, the levels of $DsbA_{SH}^{SSG}$ may be underestimated, with consequent uncertainty in both partial equilibrium constants. This is reflected in the greater variation in the values of K_1 and K_2 than of K_{eq} in Figure 7.

The values of K_{eq} and K_1 varied with pH, whereas that of K_2 was much more constant, as expected if the atypical thiol group was Cys30 of $DsbA_{SH}^{SH}$. The values of K_{eq} and K_1 decreased by nearly the expected amount between pH 4 and 6, but then changed relatively little between pH 6 and 7, before decreasing further between pH 7 and 9. Consequently, the value of K_{eq} changed only 500-fold, rather than the 10^4-fold decrease expected if only the thiol groups of Cys30 and GSH differed in pK_a values. The transition apparent in the region of pH 6–7 occurs at the same place as the transition at pH 6.7 in the reactivity of Cys30 (Figure 1), suggesting that the same phenomenon is being reflected in both. The variation of the equilibrium constants K_{eq} and K_1 would be consistent with the group titrating with pK_a 6.7 in $DsbA_{SH}^{SH}$ having a pK_a value of about 5.7 in $DsbA_{S}^{S}$ and $DsbA_{SH}^{SSG}$.

The pH dependence of K_{eq} would also include any ionization differences between GSSG and GSH, but they have been shown to be minimal (Jung et al., 1972). In confirmation, very similar values of K_{eq} were measured at pH 4, 5, and 6 by equilibrating DsbA with the thiol and disulfide forms of the very similar reagent mercaptoethanol, which has no ionizing groups. Its mixed disulfide with DsbA was not resolved by reverse-phase HPLC, however, so the partial equilibrium constants K_1 and K_2 could not be determined, and the apparent value of K_{eq} would be less accurate.

DISCUSSION

The two cysteine residues of DsbA are markedly different in their chemical properties in the native conformation, whereas they have similar and normal properties in the unfolded protein (Zapun et al., 1993, 1994) and in short peptides (Figure 1; Siedler et al., 1993). At least some of these properties are explicable in terms of the crystal structure of $DsbA_{S}^{S}$, which reveals DsbA to be structurally a member of the thioredoxin family (Martin et al., 1993). The structure of the more pertinent $DsbA_{SH}^{SH}$ is unfortunately not known but is unlikely to differ substantially from that of $DsbA_{S}^{S}$, with at most slight local changes (Zapun et al., 1993), like those demonstrated for the related thioredoxin (Dyson et al., 1990; Stone et al., 1993) and glutaredoxin (Xia et al., 1992). The thiol group of Cys30 of $DsbA_{SH}^{SH}$ is very reactive and is fully ionized, even at acidic pH values; the sulfur atom is fully exposed in the structure of $DsbA_{S}^{S}$. In contrast, the thiol group of Cys33 is very unreactive and ionizes less readily than normal; its sulfur atom is buried within the folded conformation of $DsbA_{S}^{S}$.

The apparent pK_a value of Cys30 of $DsbA_{SH}^{SH}$ is about 3.5, markedly lower than the usual value of about 8.7. Low pK_a values of thiol groups are usually due to the close proximity of positive charges. His32 is near Cys30 in the crystal structure of $DsbA_{S}^{S}$ (Martin et al., 1993), but is unlikely to be the group responsible, due to the pH dependence of the equilibrium between $DsbA_{SH}^{SH}$ and $DsbA_{S}^{S}$ (Figure 7). Any positively charged group that decreased the pK_a of Cys30 from 8.7 to 3.5 would have to have its own pK_a value increased by over 5 pH units. If this group had a pK_a value in the range 4–9 in either $DsbA_{SH}^{SH}$ or $DsbA_{S}^{S}$, its ionization state would be changed by removal of the Cys30 thiolate anion upon forming the DsbA disulfide bond and there would be no difference in net charge of $DsbA_{SH}^{SH}$ and $DsbA_{S}^{S}$ in this pH range. The marked pH dependence of the equilibrium between them (Figure 7) demonstrates that these two forms of the protein differ by about 1 net charge over the pH range 4–9 (except for the region of pH 6–7, see below). Therefore, it is unlikely that any positive charge responsible for the low pK_a value of Cys30 is that of an amino or imidazole group.

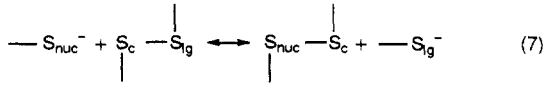
All characterized members of the thioredoxin family have low pK_a values for the corresponding thiol group: a value of about 3.5 has also been measured for glutaredoxin (Gan et al., 1990; Yang & Wells, 1991; Mieyal et al., 1991), and that of thioredoxin is no greater than 6.7 (Kallis & Holmgren, 1980). These proteins have widely divergent amino acid sequences, but similar three-dimensional folds, and some common aspect of their structures produces a thiol group with a very low pK_a value. The only apparent candidates for a positively charged group to lower the pK_a of Cys30 in the structure of $DsbA_{S}^{S}$ (Martin et al., 1993) are the peptide dipoles at the N-terminus of the α-helix on which Cys30 is

situated and which is common to the thioredoxin fold. The likely effect of the positive charge of the macro dipole of the α-helix (Wada, 1976), or the individual dipoles of peptide units at its ends (Aqvist et al., 1991; He & Quiocho, 1993), on the ionization and reactivity of the corresponding cysteine residue of thioredoxin has been emphasized by Hol (1985) and by Katti et al. (1990); Epp et al. (1983) had earlier pointed this out for the corresponding selenocysteine residue of the related glutathione peroxidase. Such dipoles would not titrate over the pH range 4–9 and would be compatible with the pH dependence of the equilibrium between DsbA and glutathione (Figure 7). In any case, the active site of the thioredoxin fold seems designed to stabilize a single thiolate anion. In $DsbA_{SH}^{SH}$ the ionized group would be Cys30. In a mixed disulfide with another molecule RSH, $DsbA_{SH}^{SSR}$, it would be Cys33; this would explain why, even though it is buried, Cys33 can ionize with a pK_a of about 9.5 and can rapidly react with the mixed disulfide of Cys30, to form $DsbA_S^S$.

Besides having a very low pK_a value, the reactivity of Cys30 was affected significantly by another group titrating with a pK_a of about 6.7 in $DsbA_{SH}^{SH}$ (Figure 1). Similar changes in the reactivities of a thiol group as a function of pH have been observed in the cases of thioredoxin (Kallis & Holmgren, 1980), thiol-subtilisin (Polgár et al., 1973; Mellor et al., 1993), and papain (Polgár & Halász, 1978), where the ionization of adjacent histidine residues has been implicated. A pK_a value of 6.7 might also suggest a histidine residue for the responsible group in DsbA, such as His32. It is undoubtedly pertinent, however, that the pH dependence of the equilibrium between $DsbA_{SH}^{SH}$ and $DsbA_S^S$ has a plateau in this pH region (Figure 7), indicating that the two forms of the protein had no difference in net charge over this pH range. The most plausible explanation is that the group titrating at pH 6.7 in $DsbA_{SH}^{SH}$ has a pK_a value of about 5.7 in $DsbA_S^S$ and $DsbA_{SH}^{SSG}$. In this case the effective pK_a of Cys30 in $DsbA_{SH}^{SH}$ would be about 4.5 at pH >6.7 and 3.5 at low pH. A carboxyl group with a somewhat elevated pK_a value is also a possible candidate for a group titrating at pH 5.7. Just such a buried aspartic acid residue has been identified in thioredoxin (Langsetmo et al., 1991); the corresponding residue in DsbA is Glu24, which is also buried (Martin et al., 1993).

The reactivity of Cys30 of $DsbA_{SH}^{SH}$ was also affected by the replacement of Cys33 by Ser (Figure 1), and the ionization of the two thiol groups seems to be mutually exclusive. This was apparent by the observation that Cys33 ionized with a pK_a value of about 9.5 in the absence of Cys30 (Figure 4d), but not at all up to pH 10 in its presence (Figure 4a). The effective pK_a of Cys33 may also be affected by the group titrating with pK_a value 6.7 in $DsbA_{SH}^{SH}$ (Figure 1).

The pK_a values of thiol groups are crucial for their reactivity. This determines the extent of thiol ionization at any particular pH and, consequently, its reactivity in most reactions. But the pK_a value of the thiol group also reflects the intrinsic chemical reactivity of the sulfur atom, including its reactivity when part of a disulfide bond. The rate of thiol–disulfide exchange between the same ionized thiol group and different disulfide bonds was observed to be directly proportional to the tendency of the resulting thiol groups to ionize (Creighton, 1975). A thorough, systematic study of the thiol–disulfide exchange reaction by Szajewski and Whitesides (1980) and Houk et al. (1987) has found the rate of the reaction



(7)

to be well-defined by the pK_a values of the three sulfur atoms when in the thiol form. At 25 °C, the value of k_{s^-} (in units of s^{-1} M^{-1}) is given by

$$\log k_{s^-} = 4.5 + 0.59\ pK_{nuc} - 0.40\ pK_c - 0.59\ pK_{lg} \quad (8)$$

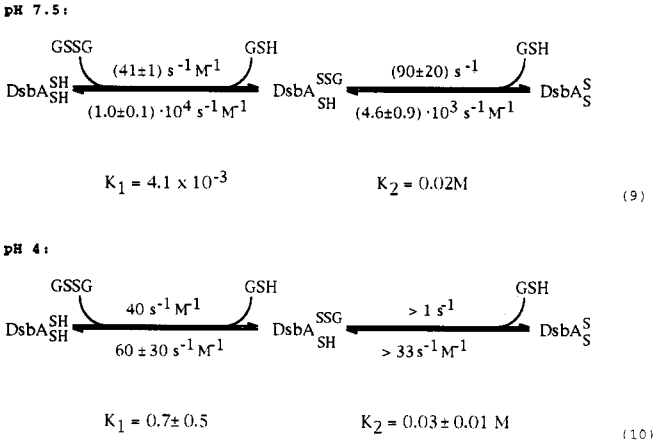
This equation can be used to calculate the rate constant at any pH for reaction between sulfur atoms with known pK_a values. It should be noted that these effects of pK_a values apply at all pH values, not just in the pH range where the thiol groups titrate, and the effective pK_a value of a thiol group can be altered even if changes in ionization of the thiol cannot be observed.

These considerations are likely to apply to DsbA and to other members of the thioredoxin family, although additional factors will also pertain, such as binding of the target molecule to the protein and steric constraints on the accessibilities of the cysteine residues. In the case of DsbA, no binding interactions with GSH or GSSG were apparent from their kinetics of reaction (Zapun et al., 1993), and the relative accessibilities of the cysteine residues are known (Martin et al., 1993). External thiol and disulfide groups are sterically restricted to reacting only with Cys30, and Cys33 can react only with the sulfur atom of Cys30. In the absence of these steric restrictions, an external thiol group would be expected to react preferentially with the sulfur atom of a disulfide bond with the higher pK_a, Cys33.

The observed rate constants of the bimolecular reactions between DsbA and glutathione can be compared with those expected on the basis of eq 8 with normal and low pK_a values for Cys30. At pH 7.5, the effective pK_a of Cys30 might be about 4.5, due to the effect of the group ionizing at pH 6.7, but the following analysis would be even more dramatic if a pK_a of 3.5 were used. The effect of a pK_a of 4.5, rather than the normal 8.7, for Cys30 should be to increase the rate of reaction of GSH with $DsbA_S^S$ about 50-fold; the reverse, intramolecular reaction should also be increased in rate by the same amount, and the equilibrium constant K_2 for this step should be unchanged. The reaction of the mixed disulfide $DsbA_{SH}^{SSG}$ with GSH should be increased about 300-fold, because Cys30 is a very favorable leaving group, while the reverse reaction of GSSG with Cys30 should be decreased by a factor of about 0.06. Consequently, the equilibrium constant K_1 and the overall equilibrium constant K_{eq} should be decreased by about a factor of 2×10^{-4}. Therefore, the stabilities of the disulfide bonds of $DsbA_{SH}^{SSG}$ and $DsbA_S^S$ are decreased by this factor simply as a result of the low pK_a value of Cys30. This suggests that one factor in the instabilities of these disulfide bonds may be electrostatic interactions of the Cys30 thiol group in $DsbA_{SH}^{SH}$ to produce a low pK_a value.

With an effective pK_a value of about 4.5 in the folded state and 8.7 in the unfolded state at neutral pH, the thiolate anion of Cys30 should stabilize the native conformation by about 5.7 kcal/mol. Consequently, removing the thiolate anion should destabilize the native conformation. In agreement, although only qualitatively, the folded conformations of $DsbA_{SH}^{OH}$, $DsbA_{OH}^{SSG}$, and $DsbA_S^S$ have been found to be respectively 1.8, 1.5, and 4 kcal/mol less stable than that of the corresponding reduced protein when the Cys30 thiol group is present (Zapun et al., 1993, 1994). These discrepancies, plus the observation that glutaredoxin also has a thiol group with a very low pK_a value (Gan et al., 1990; Yang & Wells, 1991; Mieyal et al., 1991), but a much more stable disulfide bond than DsbA (Sandberg et al., 1991), indicate that other factors also determine the stability of the thioredoxin motif disulfide bond.

The low pK_a value of the Cys30 thiol group can largely explain its ability to transfer disulfide bonds directly into reduced proteins at acidic pH (Wunderlich et al., 1993; Zapun & Creighton, 1994). Thiol–disulfide exchange between normal sulfur atoms is usually 3000-fold slower at pH 4 than at pH 7.5, due to the decreased ionization of the thiol group. Instead, the rates of reaction between DsbA and glutathione are not markedly different at pH 4 (eq 6) and at pH 7.5 (Zapun et al., 1993):



None of the rates are decreased by the factor of 3000-fold that would be expected with normal thiol groups. That the rate of reaction between $DsbA^{SH}_{SH}$ and GSSG is the same at pH 7.5 and 4 is readily explained by the full ionization of Cys30 of $DsbA^{SH}_{SH}$ at both pH values, but the large values of the other rate constants at pH 4 are initially more surprising. For example, the thiol group of GSH (with a pK_a value of about 8.8) should be 3000-fold less ionized at pH 4 than at pH 7.5, yet the rates of its reaction with $DsbA^{S}_{S}$ and with $DsbA^{SSG}_{SH}$ are slowed no more than 200-fold. This appears to be due to the effect of the putative group ionizing with pK_a values of about 6.7 and 5.7 in $DsbA^{SH}_{SH}$ and $DsbA^{S}_{S}$, respectively. One of its effects is to diminish the pH dependence of the equilibrium constant between the two forms of the protein and glutathione to less than would otherwise occur (Figure 7). The forward rate constant for the reaction between GSSG and $DsbA^{SH}_{SH}$ is the same at pH 4 and 7.5, so the reverse rate constant must decrease less at acidic pH than would otherwise occur.

The rates of the bimolecular reactions between DsbA and glutathione at neutral and acidic pH are all about 10^2 times greater than would be predicted by eq 8. It is likely, therefore, that DsbA catalyzes the rates of all the thiol–disulfide exchange reactions at its active site. This could be a further consequence of the active site of DsbA stabilizing the presence of a single negative charge. The rates of all the reactions would be increased by stabilizing the transition state, where the negative charge is spread over three sulfur atoms (eq 7).

A similar situation is observed in the thioredoxin motif of glutathione transferases, where the cysteine thiol group also has a very low pK_a value (Lo Bello et al., 1993) and where that of bound GSH is lowered to about 6.2 (Graminski et al., 1989). This has been attributed to hydrogen bonding to a tyrosine residue, but it also has a greatly lowered pK_a value (Atkins et al., 1993; Karshikoff et al., 1993; Meyer et al., 1993), which would indicate that this enzyme also stabilizes a negative charge in its active site.

The pK_a values of the cysteine thiol groups are clearly crucial to understanding the thiol–disulfide exchange properties of DsbA and other proteins of the thioredoxin family, and the relationship of eq 8 from Szajewski and Whitesides (1980) suggests that it may be possible to understand the rates of the reactions also. Using site-directed mutagenesis and the linked functions of group ionization, disulfide bond stability, and folded conformation stability, it should be possible to understand the functions of DsbA, PDI, thioredoxin, and the other members of this family. One major question to be answered is why the stabilities of the disulfide bonds of DsbA and thioredoxin differ by 6.9 kcal/mol at neutral pH even though they are structurally indistinguishable (Martin et al., 1993; Wunderlich & Glockshuber, 1993; Zapun et al., 1993).

ACKNOWLEDGMENT

We are grateful to Monique van Straaten for assistance; J. C. A. Bardwell for providing the strains to produce DsbA and for much advice and encouragement; R. Glockshuber for communicating results prior to publication; J. Martin and J. Kuriyan for providing the coordinates of the $DsbA^{S}_{S}$ structure prior to publication; D. Nalis and R. Jacob for synthesizing and purifying the peptide; E. Penka and A. Zapun for providing the purified proteins; and N. J. Darby and A. Zapun for much advice and many stimulating discussions. J.W.N. was supported in part by a Louisiana State University Council on Research Summer Stipend.

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