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Redox properties of protein disulfide isomerase (DsbA) from *Escherichia coli*

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Abstract

The redox properties of periplasmic protein disulfide isomerase (DsbA) from *Escherichia coli* were analyzed by measuring the equilibrium constant of the oxidation of reduced DsbA by oxidized glutathione. The experiments are based on the finding that the intrinsic tryptophan fluorescence of DsbA increases about threefold upon reduction of the enzyme, which can be explained by the catalytic disulfide bridge quenching the fluorescence of a neighboring tryptophan residue. From the specific fluorescence of DsbA equilibrated in the presence of different ratios of reduced and oxidized glutathione at pH 7, an equilibrium constant of 1.2×10^{-4} M was determined, corresponding to a standard redox potential (E_0') of DsbA of −0.089 V. Thus, DsbA is a significantly stronger oxidant than cytoplasmic thioredoxins and its redox properties are similar to those of eukaryotic protein disulfide isomerase. The equilibrium constants for the DsbA/glutathione equilibrium were found to be strongly dependent on pH and varied from 2.5×10^{-3} M to 3.9×10^{-5} M between pH 4 and 8.5. The redox state-dependent fluorescence properties of DsbA should allow detailed physicochemical studies of the enzyme as well as the quantitative determination of the oxidized protein by fluorescence titration with dithiothreitol and open the possibility to observe bacterial protein disulfide isomerase "at work" during catalysis of oxidative protein folding.

Keywords: disulfide interchange; dithiothreitol; DsbA protein; *Escherichia coli*; glutathione; protein disulfide isomerase; redox potential

The formation of disulfide bonds in the periplasmic space of *Escherichia coli* is catalyzed by the DsbA protein (Bardwell et al., 1991; Kamitani et al., 1992). This recently discovered PDI consists of 189 residues with two cysteines comprising the catalytic disulfide bridge. The enzyme does not exhibit an overall sequence homology to cytoplasmic thioredoxin of *E. coli* and the well-characterized eukaryotic PDI located in the ER. However, DsbA shares a common active site with oxidoreductases such as PDI, thioredoxin, and glutaredoxin, as the active cysteines are separated by only two residues and the amino acid sequences surrounding the active disulfide are similar in all these enzymes (Bardwell et al., 1991).

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Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; FPLC, fast performance liquid chromatography; GdmCl, guanidinium chloride; GSH, reduced glutathione; GSSG, oxidized glutathione; IPTG, isopropyl-β-D-thiogalactoside; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; NAD^+, nicotinamide adenine dinucleotide; PDI, protein disulfide isomerase; SDS, sodium dodecyl sulfate.

The intrinsic redox potentials of thioredoxins and eukaryotic PDI have been determined and were found to be in the range of −0.26 V to −0.23 V and −0.11 V, respectively (Holmgren, 1968; Berglund & Sjöberg, 1970; Hawkins et al., 1991; Gleason, 1992). Therefore, eukaryotic PDI is a stronger oxidant than thioredoxin. This is consistent with the localization and function of both enzymes in the cell. Eukaryotic PDI is located in the lumen of the ER, where it catalyzes oxidative protein folding, i.e., the formation of disulfide bonds of secretory proteins (Bulleid & Freedman, 1988; Freedman, 1989; Noiva & Lennarz, 1992). Thioredoxin occurs in the cytoplasm of bacterial and eukaryotic cells where the redox conditions are reducing and where the protein is involved in regulation of protein activity by catalysis of NADPH-dependent reductions of functional disulfide bonds (Holmgren, 1985, 1989). In plants, thioredoxin is also known to be a regulator of photosynthetic enzymes (Cséke & Buchanan, 1986).

In vitro experiments on the mechanism of oxidative protein folding catalyzed by eukaryotic PDI using reduced RNase A as a substrate have revealed that PDI-me-

diated folding strongly depends on the redox conditions, i.e., the ratio of oxidized and reduced thiol agents such as GSH and GSSG. In addition, the catalytic effect of PDI on RNase A folding was found to be maximal at the redox conditions optimal for uncatalyzed folding of RNase A (Hawkins et al., 1991; Lyles & Gilbert, 1991). The proposed mechanism of PDI-catalyzed folding involves mixed PDI–protein disulfide intermediates as well as thiol–disulfide interchange reactions with GSH/GSSG of both the folding polypeptide and PDI (Saxena & Wetlaufer, 1970; Hawkins et al., 1991; Lyles & Gilbert, 1991; Lundström et al., 1992). In this context, the intrinsic redox potential of PDI determines the relative amounts of oxidized and reduced PDI at equilibrium under the chosen redox conditions, which may affect the velocity of PDI-catalyzed protein folding independently of the reaction rate of uncatalyzed oxidative folding.

Unlike in the cytoplasm and in the lumen of the ER, the redox conditions in the periplasmic space of *E. coli* may be varying and are likely to be not regulated. They mainly depend on the conditions of bacterial growth (which may be aerobic or anaerobic) and the fact that small oxidizing and reducing molecules with a molecular mass below 500 Da can freely pass the outer membrane of *E. coli* (Payne & Gilvarg, 1968; Decad & Nikaido, 1976). As a result, the ability of periplasmic protein disulfide isomerase of *E. coli* (DsbA) to catalyze oxidative protein folding in vivo may strongly depend on the redox conditions in the periplasm.

The purpose of the present investigation was to determine the intrinsic redox properties of bacterial PDI, which may reflect the environmental conditions where this enzyme is essential for disulfide formation in the periplasm. The method to analyze the redox state of DsbA is based on the fluorescence emission properties of the protein, which are remarkably different in the reduced and the oxidized state of the enzyme.

Results

Expression and purification of DsbA

To obtain bacterial PDI in high amounts from *E. coli*, the *dsbA* gene was cloned and overexpressed. The gene was amplified from the genome of the *E. coli* K12 wild-type strain W3110 (Bachmann, 1972) by the polymerase chain reaction using oligonucleotide primers based on the published *dsbA* sequence (Bardwell et al., 1991). The complete gene including its natural signal sequence and its natural ribosomal binding site was cloned into a derivative of the expression plasmid pASK40, where the *dsbA* gene is under the control of the lac promoter/operator (Skerra et al., 1991). The gene was sequenced and found to be identical with the published *dsbA* sequence (Bardwell et al., 1991) except for one silent mutation in the codon of residue Ala-143 in the mature DsbA protein (GCA instead of GCT). The *E. coli* K12 strain JM83 (Yanisch-Perron et al., 1985) was transformed with the resulting expression plasmid. Upon induction with IPTG, the cells produced large amounts of functional DsbA, which was found to be the most abundant protein in the periplasmic fraction (Fig. 1). DsbA was purified from the periplasmic extract of induced cells in the absence of reducing agents by sequential chromatography on DE52 cellulose and phenyl superose. As estimated from densitometric analysis of SDS–polyacrylamide gels, the protein was 90% pure after the first chromatographic step. Subsequent chromatography on phenyl superose typically yielded 7 mg of homogeneous DsbA per liter of bacterial culture (Fig. 1).

The oxidation state of DsbA obtained by the outlined purification procedure was investigated by analysis of free sulfhydryl groups using Ellman's reagent (DTNB), assuming that the disulfide exchange equilibrium between SDS-denatured DsbA and DTNB lies far on the side of reduced DTNB (Ellman, 1959). Less than 0.10 μM thiol groups could be titrated at a protein concentration of 50 μM, sug-

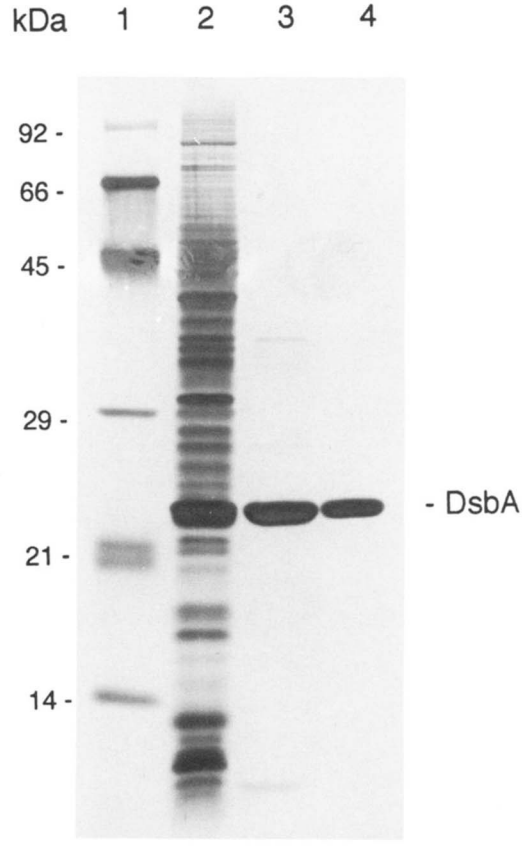

Fig. 1. Purification of DsbA. A silver-stained 15% SDS–polyacrylamide gel is shown. Lane 1, molecular mass standard; lane 2, periplasmic extract from induced *E. coli* cells; lane 3, after chromatography on DE52 cellulose; lane 4, purified DsbA after chromatography on phenyl superose. A detailed description of the purification procedure is given in the Materials and methods section.

gesting that all DsbA molecules were obtained in the oxidized form.

Fluorescence properties of DsbA

Starting with the observation that the fluorescence emission spectra of thioredoxin are strongly dependent on the redox state of the enzyme (Holmgren, 1972; Berglund & Holmgren, 1975; Gleason, 1992), the fluorescence emission spectra of reduced and oxidized DsbA were compared. DsbA shows a 3.2-fold increase in fluorescence intensity at 322 nm at pH 7.0 after complete reduction of the oxidized form with DTT, when the protein is excited at 280 nm, i.e., when tyrosine and tryptophan residues are excited simultaneously. An identical relative increase in fluorescence emission is obtained, when only tryptophan residues are excited (excitation wavelength: 295 nm). Therefore, at least one of the two tryptophan residues of the protein may be located next to the catalytic disulfide, which in turn quenches the tryptophan fluorescence. The DsbA emission maximum (322 nm) was unchanged in the oxidized and reduced form and independent of the excitation wavelength. In the presence of 6 M GdmCl identical spectra were obtained for oxidized and reduced DsbA with emission maxima at 350 nm, which are typical for denatured proteins (Fig. 2). This is consistent with the as-

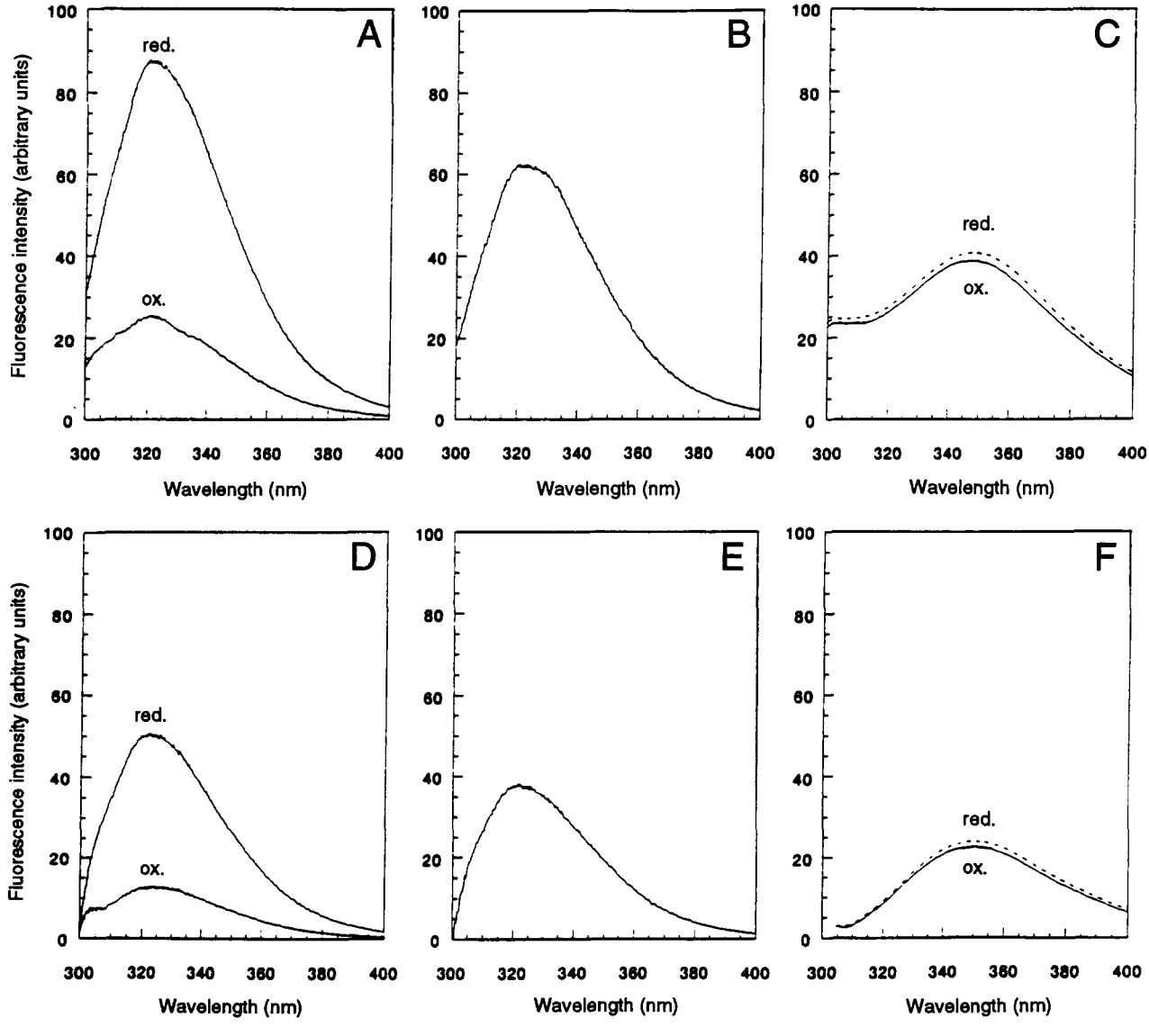

Fig. 2. Fluorescence emission properties of oxidized and reduced DsbA. Fluorescence emission spectra were recorded at protein concentrations of 1.0 μM in 100 mM sodium phosphate, pH 7.0, 1 mM EDTA. Samples of reduced DsbA contained DTT (10 mM) and samples of unfolded DsbA contained GdmCl (6 M). **A, D:** Fluorescence spectra of oxidized and reduced DsbA. **B, E:** Difference spectra. **C, F:** Fluorescence spectra of unfolded oxidized and reduced DsbA in the presence of 6 M GdmCl. A–C: Excitation at 280 nm. D–F: Excitation at 295 nm. Note that the scale of the fluorescence intensity is the same in A–F.

sumption that a tryptophan must be in the region of the catalytic disulfide. Unlike in thioredoxin, none of the tryptophan residues is close to the disulfide in the DsbA sequence. Therefore, one or both tryptophans become located next to the disulfide as a consequence of the three-dimensional structure of the DsbA protein, and thus the disulfide has no influence on the emission properties of unfolded DsbA.

Like thioredoxin (Stryer et al., 1967; Holmgren, 1972; Reutimann et al., 1981; Gleason, 1992), the reduced form of DsbA shows a pH-dependent fluorescence signal, whereas the fluorescence of the oxidized form is unaffected by changes in pH (Fig. 3). However, the differences in the emission of reduced DsbA are small, and thus no conclusions can be drawn on pK_a values of residues in the area of the tryptophans and the catalytic disulfide bridge. The fact that the emission maximum of DsbA is unchanged between pH 4 and 9 provides evidence that the oxidized and reduced forms of DsbA do not undergo large conformational changes or denaturation and are fully stable in this pH range.

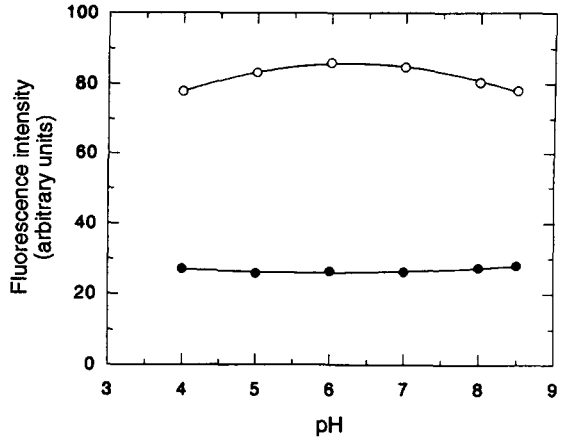

Fig. 3. Dependence of DsbA fluorescence on pH at 30 °C. The fluorescence intensities of DsbA (2.0 μM) at 322 nm were recorded for 1 min and averaged (excitation at 280 nm). The following buffers were used: pH 4.0, 100 mM formic acid/NaOH; pH 5.0, 100 mM acetic acid/NaOH; pH 6.0–7.0, 100 mM sodium phosphate; pH 8.0–8.5, 100 mM Tris/HCl. Reduced DsbA was obtained after incubation of DsbA in the presence of 10 mM DTT for 30 min at 30 °C. Buffers were shown to have no influence on DsbA fluorescence. ○, reduced DsbA; ●, oxidized DsbA.

Fluorescence titration with DTT

In our first attempt to determine the redox properties of bacterial protein disulfide isomerase, we used the specific fluorescence of DsbA to measure the equilibrium concentrations of the oxidized and the reduced form in the presence of different ratios of oxidized (DTT_{ox}) and reduced DTT. The equilibrium for the oxidation of reduced protein disulfide isomerase (PDI_{red}) by oxidized DTT and its equilibrium constant are given by Equations 1 and 2:

$$PDI_{red} + DTT_{ox} \overset{K_{eq}}{\rightleftharpoons} PDI_{ox} + DTT \quad (1)$$

$$K_{eq} = [PDI_{ox}][DTT]/[PDI_{red}][DTT_{ox}]. \quad (2)$$

Starting with oxidized DsbA, the specific fluorescence of the protein at pH 7.0 and 30 °C was measured as a function of increasing amounts of DTT. The resulting titration curve is characterized by a linear increase in fluorescence intensity at 322 nm, which becomes constant after the addition of 0.97 molar equivalents DTT, as determined from the intersection of the linear regression lines (Fig. 4A). This corresponds to the expected DTT/DsbA ratio of 1:1 and lies within the experimental error of determining the protein concentration (Gill & von Hippel, 1989). The fully oxidized state of the DsbA preparation could thus be confirmed. The experiment also demonstrated that the DsbA/DTT equilibrium lies far on the side of reduced PDI.

In order to determine the equilibrium constant of the DsbA/DTT redox system, we then used mixtures of DTT_{ox} and reduced DTT. The titration experiment described above was repeated in the presence of 0.1 mM DTT_{ox}. However, an almost identical titration profile was obtained and again no oxidized DsbA could be detected at equilibrium, when DTT was present in a molar excess (Fig. 4B). Due to the strong absorbance of oxidized DTT at 280 and 295 nm at concentrations above 0.1 mM, which interferes with the fluorescence experiment, the $[DTT_{ox}]/[DTT]$ ratio could not be increased essentially further. Thus, no experimental conditions for the determination of K_{eq} using the intrinsic DsbA fluorescence could be established. However, we could estimate that the value of K_{eq} was 10^{-5} or less, since no oxidized DsbA could be detected when DTT was present in equimolar amounts and DTT_{ox} in a 200-fold excess.

The pH-dependent redox equilibrium with GSH/GSSG

Because the DTT/DTT_{ox} system had turned out to be unsuitable for measuring a redox equilibrium with DsbA, we analyzed the redox equilibrium of DsbA with GSH and GSSG. The glutathione redox couple has a higher standard redox potential than the DTT couple at 30 °C and pH 7 (−0.205 V compared with −0.323 V, respectively; Szajewski & Whitesides, 1980) and should thus allow experimental conditions to determine the DsbA/glutathione redox equilibrium, which is given by Equations 3 and 4:

$$PDI_{red} + GSSG \overset{K_{eq}}{\rightleftharpoons} PDI_{ox} + 2GSH \quad (3)$$

$$K_{eq} = [PDI_{ox}][GSH]^2/[PDI_{red}][GSSG]. \quad (4)$$

When DsbA was incubated in the presence of 0.1 mM GSSG and different concentrations of GSH (0.001–2 mM)

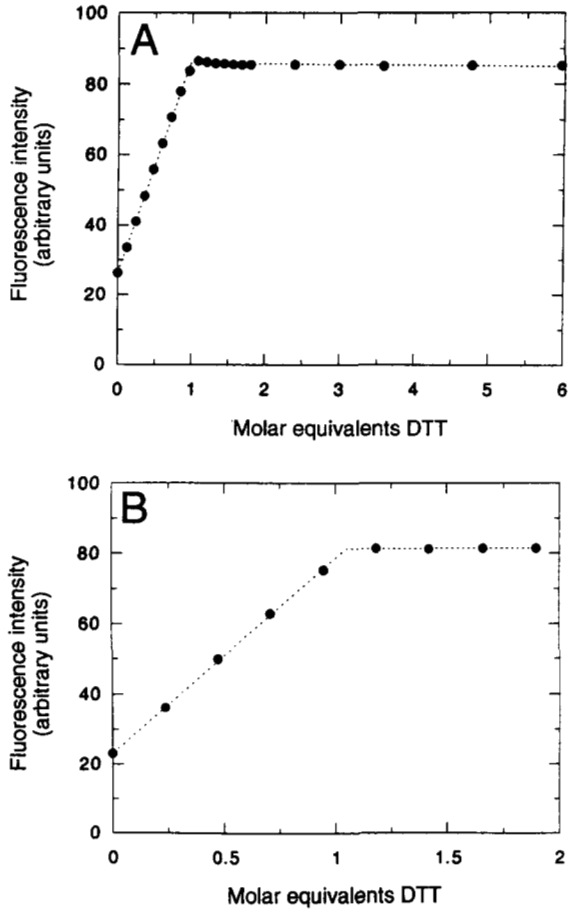

Fig. 4. Fluorescence titration of oxidized DsbA with DTT. The reduction of oxidized DsbA by DTT was detected using the change in the specific DsbA fluorescence at 322 nm (excitation at 280 nm). **A:** A solution of DTT (10 μM) was added stepwise to a solution of oxidized DsbA (0.84 μM) in 100 mM sodium phosphate, pH 7.0, 1 mM EDTA. Ten minutes after each addition, the fluorescence at 322 nm was recorded for 1 min, averaged, and corrected for the volume increase. **B:** As described under A, but in the presence of oxidized DTT (0.1 mM) and at a protein concentration of 0.42 μM.

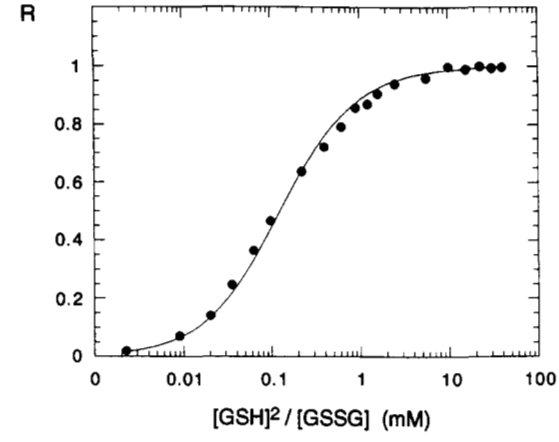

Fig. 5. Redox equilibrium of DsbA with glutathione. The relative amount of reduced DsbA at equilibrium (R) was measured using the specific DsbA fluorescence at 322 nm (excitation at 280 nm). Oxidized DsbA (0.42 μM) was incubated for 12 h in 100 mM sodium phosphate, pH 7.0, 1 mM EDTA, containing 0.1 mM GSSG and different concentrations of GSH (0–2,000 μM). The equilibrium concentrations of oxidized and reduced DsbA, GSH and GSSG were calculated according to Equations 9–11. The equilibrium constant (K_{eq}) was determined by fitting the data according to Equation 5 (Hawkins et al., 1991). After nonlinear regression, a value of 1.2×10^{-4} M was obtained (correlation coefficient: 0.9993).

under exclusion of oxygen, the relative amount of reduced DsbA at equilibrium (R) could indeed be measured over the whole range from the oxidized to the reduced protein using the specific DsbA fluorescence (Fig. 5). By plotting R against the $[GSH]^2/[GSSG]$ ratio (Equation 5; Hawkins et al., 1991), the equilibrium constant K_{eq} for the DsbA/glutathione system was found to be 1.2×10^{-4} M after nonlinear regression (Fig. 5). The standard redox potential of DsbA at pH 7 and 30 °C ($E'_{0\,PDI}$) was then calculated from the Nernst equation using the glutathione standard potential ($E'_{0\,GSH/GSSG}$ at pH 7 and 30 °C; Equation 6) and found to be −0.089 V.

$$R = \frac{[GSH]^2/[GSSG]}{K_{eq} + [GSH]^2/[GSSG]}, \qquad (5)$$

$$E_{0\,PDI} = E_{0\,GSH/GSSG} - (RT/nF) \times \ln K_{eq}. \qquad (6)$$

Thus, the intrinsic redox potential of DsbA is similar to that of eukaryotic PDI (−0.11 V; Hawkins et al., 1991) and significantly different from the values obtained for cytoplasmic thioredoxins ranging from −0.23 to −0.26 V (Holmgren, 1968; Berglund & Sjöberg, 1970; Gleason, 1992).

We then investigated the pH dependence of the DsbA/glutathione equilibrium at 30 °C between pH 4 and 8.5. The equilibrium was found to be strongly dependent on pH. The K_{eq} values decreased within two orders of magnitude from 2.5×10^{-3} M at pH 4 to 3.9×10^{-5} M at pH 8.5 (Table 1). As expected from the dependence of disulfide exchange kinetics on pH, the DsbA/glutathione equilibrium at pH 8.5 was reached within several minutes after mixing of oxidized DsbA with different concentrations of GSH and GSSG, whereas incubation times of 3 days were necessary at pH 4, until reproducible values of K_{eq} were obtained (Table 1).

To determine the standard redox potentials of DsbA from the equilibrium constants, the standard potentials of the GSH/GSSG couple were calculated at each pH. Based on the fact that the pK_a values of the carboxylate groups and the amino group of glutathione are nearly identical in the oxidized and reduced form (Jung et al., 1972), the E_0^{pH} values for the glutathione couple at 30 °C were calculated from the known standard potential at pH 7 (E'_0) according to the general equation for the pH

Table 1. *Dependence of DsbA/glutathione equilibrium constants and DsbA redox potentials on pH at 30 °C*[a]

	pH				
	4.0	5.0	6.0	7.0	8.5
K_{eq} (M)	2.5×10^{-3}	6.7×10^{-4}	2.5×10^{-4}	1.2×10^{-4}	3.9×10^{-5}
$E_{0\,GSH/GSSG}$ (V)	−0.024	−0.085	−0.145	−0.205	−0.295
$E_{0\,DsbA}$ (V)[b]	0.053	0.009	−0.039	−0.089	−0.165
Time of incubation	72 h	24 h	18 h	12 h	20 min

[a] The E_0^{pH} values for the glutathione couple at 30 °C were calculated from the equation $E_0^{pH} = E_0' - (RT/nF) \times 2.303 \times 2 \times (pH - 7)$, which considers the involvement of two protons in the GSH/GSSG potential. E_0' is the standard potential of the GSH/GSSG couple at 30 °C and pH 7 (−0.205 V), R is the gas constant (8.315 J K^{-1} mol^{-1}), T is the absolute temperature (303.15 K), n is the number of transferred electrons (2), and F is the Faraday constant (9.649×10^4 C mol^{-1}). Using these parameters, Equation 7 is obtained. Experimental conditions are given in the Materials and methods section.

[b] Standard potentials for DsbA were calculated according to Equation 6 using the calculated E_0^{pH} values for the glutathione couple.

dependence of redox potentials involving two protons (Equation 7; see Table 1).

$$E_0^{pH} = E_0' - 60.2\ mV \times (pH - 7). \quad (7)$$

Thus, the E_0 value of the GSH/GSSG couple changes for 60.2 mV with each pH unit. Obviously, the determined pH dependence of the DsbA standard potential does not correspond to this model. Although a linear correlation between pH and $E_{0\,DsbA}$ is observed (correlation coefficient: 0.9996), the E_0 values changed by only 48.6 mV per pH unit. Therefore, DsbA becomes a weaker oxidant for GSH with decreasing pH and a stronger oxidant with increasing pH (Table 1). The deviation from the simple model for the pH dependence of E_0 may represent contributions of charged residues near the catalytic disulfide of the DsbA molecule as well as different pK_a values of both cysteine residues.

Discussion

Fluorescence properties of DsbA as a quantitative assay for DsbA and a tool for studying its function

The remarkably different fluorescence emission properties of oxidized and reduced DsbA are very useful for several reasons: First, the fact that the redox equilibrium of DsbA with DTT lies far on the side of the reduced enzyme (see below) allows the quantitative determination of oxidized DsbA at low protein concentration by fluorescence titration with DTT. Due to the large, about threefold increase in fluorescence emission at pH values between 4 and 8.5, fluorescence titration with DTT should be sensitive enough to determine the concentration of oxidized DsbA even in the presence of impurities. This assay may be especially useful to detect DsbA during its purification from the periplasmic *E. coli* fraction, because the protein is obtained in the fully oxidized form.

Second, the redox-dependent DsbA fluorescence can be used to measure the kinetics of oxidation and reduction of the enzyme under various conditions and to extract thermodynamic parameters from the temperature dependence of redox equilibria between organic thiols and DsbA.

Third, the fluorescence emission properties of DsbA should also allow the selective spectroscopic observation of its redox state during catalysis of oxidative protein folding in vitro: Because the fluorescence increase of DsbA is still about threefold upon reduction, when only the tryptophans are excited, the use of protein substrates that do not contain tryptophan residues may open the possibility to observe bacterial protein disulfide isomerase "at work" during oxidative folding and to correlate the time course of its fluorescence signal with the appearance and disappearance of folding intermediates and the formation of the native substrate protein.

The pH dependence of equilibrium constants and redox potentials

The pH dependence of disulfide exchange equilibria involving PDI is complicated for several reasons. First, the observed equilibrium constant K_{eq} of disulfide exchange reactions is strongly influenced by the *difference* in pK_a values of the thiol reagents, i.e., GSH and reduced PDI, and only becomes independent of pH when all pK_a values are identical (Szajewski & Whitesides, 1980). As an example, changes of four orders of magnitude in the K_{eq} value of an equilibrium involving only monothiol species are observed when the pK_a values of the thiols are two pH units apart, as the pH is changed from acidic conditions where the thiols are completely protonated to basic conditions, where the thiolate forms of both species are present. As a consequence, the pH dependence of K_{eq} is strongest around the mean value of both pK_a values (Szajewski & Whitesides, 1980). Second, the pK_a values

of both thiols of DsbA are presently unknown and unlikely to be identical neither with each other nor with the pK_a value of GSH, which is in the range of 9 (Szajewski & Whitesides, 1980). In the case of human thioredoxin, the pK_a values of both cysteine residues comprising the active disulfide (Cys-32 and Cys-35) have been measured by NMR spectroscopy and were found to be 6.3 ± 0.1 and 7.5–8.6, respectively (Forman-Kay et al., 1992). Similar results have been obtained for *E. coli* thioredoxin, where the pK_a values of the active thiols are in the range of 7.5 (Dyson et al., 1991). Therefore, reduced thioredoxin and probably also reduced DsbA may be considered as unsymmetrical dithiol reagents with pK_a values different from the pK_a of GSH. This should further complicate the expected pH dependence of the DsbA/glutathione equilibrium. Third, the pK_a values and hence the nucleophilic properties of both active cysteine residues in DsbA may also be influenced by charged neighboring residues, which in turn may titrate at abnormal values. Taken together, the pH dependence of the DsbA standard potential and K_{eq} for the DsbA/glutathione equilibrium may reflect a difference between the pK_a values of the individual DsbA thiols and of glutathione as well as the molecular environment of the thiols in the protein. Therefore, a detailed knowledge of pK_a values of residues comprising the active site of DsbA is the prerequisite for understanding the mechanism of disulfide exchange reactions involving DsbA.

Obviously, the measured pH dependence for the DsbA/glutathione equilibrium is not consistent with the expected pH dependence of a simple disulfide exchange reaction comprising two thiol species with different pK_a values (Szajewski & Whitesides, 1980). Comparison of the obtained values for K_{eq} with theoretical curves for such a simple model reveals that probably more than two thiol species contribute to the apparent equilibrium. Therefore, the concept of the DsbA molecule appears to be the prevention of large changes in K_{eq} with simultaneous use of a cysteine thiol of strongly reduced pK_a.

Accuracy and meaning of standard redox potentials

The main problem in the determination of standard redox potentials is given by the fact that E_0 values cannot be measured directly, but have to be calculated from a known E_0 of another redox couple equilibrated with the redox couple with unknown E_0. The commonly used value for the GSH/GSSG couple at pH 7.0 (E_0') is -0.24 ± 0.01 V. This value has been determined at 40 °C using the $NAD^+/NADH_2$ pair as reference and was calculated from a value of $\approx 10^3$ for the equilibrium constant (Rost & Rapoport, 1964). In this study, we have used the value of −0.205 V for the glutathione couple, which has been determined very accurately at 30 °C using the lipoamide/NAD^+ system (Szajewski & Whitesides, 1980). Because standard redox potentials are temperature dependent, all redox equilibria were measured at this temperature (30 °C). Another reason to use the value of −0.205 V for the glutathione couple is that the standard redox potentials of other commonly used thiol reagents (including the DTT/DTT_{ox} couple) have been determined under conditions identical to those in the study of Szajewski and Whitesides (1980). This allows the direct comparison of the redox equilibria of DsbA with glutathione and with DTT. Using the value of $E_0' = -0.323$ V for the DTT/DTT_{ox} couple (Szajewski & Whitesides, 1980) and the measured E_0' for DsbA (−0.089 V), K_{eq} for the DsbA/DTT equilibrium was calculated. The value of 1.2×10^{-8} is consistent with the estimated value of $K_{eq} \leq 10^{-5}$.

Using the intrinsic protein fluorescence as a measure of the redox state of DsbA, the equilibrium with different thiol/disulfide systems can be determined very accurately (±10%). Therefore, DsbA may prove a general tool to measure standard redox potentials of organic sulfhydryl reagents with an accuracy of ±0.001 V, provided a reference redox couple is used, where the potential has been determined with a similar accuracy under identical experimental conditions.

A quantitative comparison of the intrinsic redox potentials of DsbA and eukaryotic PDI cannot be made presently. Although the redox potential of eukaryotic PDI (−0.11 V) has also been determined by equilibration of the enzyme with GSH/GSSG, a standard redox potential of −0.24 V was used for the GSH/GSSG couple, and the equilibrium was investigated at 25 °C and pH 7.5 (Hawkins et al., 1991). For a semiquantitative comparison, we estimate a K_{eq} of ≈ 80 μM at pH 7.5 from the overall pH dependence of the DsbA/glutathione equilibrium (Table 1). Assuming a negligible temperature dependence of the DsbA/glutathione equilibrium between 25 and 30 °C, this value is similar to the value of 42 μM for the equilibrium with eukaryotic PDI (Hawkins et al., 1991). Therefore, the E_0' values of both enzymes appear very similar.

In vivo function of protein disulfide isomerase

Regarding the situation in vivo, i.e., the conditions in the ER of higher cells and in the periplasmic space of *E. coli*, the measured equilibrium constants for PDIs with glutathione may generally be more informative than redox potentials, since it seems unlikely that DsbA or eukaryotic PDI themselves act as oxidants or reductants. Both enzymes rather catalyze the formation and breakage of disulfide bonds in folding polypeptide chains by other oxidants and reductants such as other thiols and disulfides present in the bacterial periplasm and the ER. In the case of eukaryotic PDI, it is possible to calculate the redox state of the enzyme in vivo, because GSH and GSSG have been shown to be the molecular species that determine the thiol-dependent redox state of the ER (Hwang et al., 1992). Taking the total concentration of GSH in the ER (~1 mM), GSH/GSSG ratios between 1:1 and 3:1 (Hwang

et al., 1992), and the equilibrium constant at pH 7.5 of 42 μM (Hawkins et al., 1991), the calculated ratio of PDI_{ox}/PDI_{red} in vivo varies between 0.01 and 0.04, when we assume a pH of 7.5 in the ER lumen (although the pH in the ER is presently unknown, it seems plausible that it is regulated and not significantly different from this value).

At the first view, the calculated, 25–100-fold excess of PDI_{red} over PDI_{ox} suggests that catalysis of reduction of non-native disulfides in a folding polypeptide chain is the predominant role of eukaryotic PDI in vivo. This has also been proposed from the in vitro experiments on PDI-catalyzed folding of RNase A (Lyles & Gilbert, 1991). However, the following consideration, which is valid for both bacterial and eukaryotic PDI due to their almost identical standard potentials, indicates that this is probably not the case. The equilibrium for the oxidation of a reduced, folding polypeptide chain (P_{red}) by PDI is given by Equation 8:

$$P_{red} + PDI_{ox} \underset{k_{red}}{\overset{k_{ox}}{\rightleftharpoons}} P_{ox} + PDI_{red} \cdot \quad (8)$$

Assuming a redox potential of about −0.2 V for a reduced, folding polypeptide chain, which is likely to be in the range of monothiol species such as glutathione (Szajewski & Whitesides, 1980), and taking a mean value of −0.1 V for bacterial and eukaryotic PDI, an equilibrium constant K_{eq} in the range of 10^{-3} to 10^{-4} is obtained according to the Nernst equation. Therefore, the rate constant for the oxidation of the polypeptide chain (k_{ox}) by PDI would be 10^3–10^4 times higher than the rate constant for the reduction of a disulfide in the polypeptide (k_{red}). Including the excess of reduced PDI over the oxidized form, the oxidation of a single polypeptide chain by PDI would still be 10–400 times faster than its reduction. Therefore, it appears that the oxidation of a folding protein is the predominant role of protein disulfide isomerases. Consequently, it seems plausible that the reduced form of PDI is present in a molar excess over the oxidized form, because this excess may be the prerequisite of the occurrence of the reduction of non-native disulfides in a significant amount. In summary, the ratio of oxidized and reduced PDI in the ER (and probably also in the bacterial periplasm) may indeed reflect the need for catalysis of formation and—to a smaller extent—reduction of disulfide bonds in folding proteins. In contrast to the conditions in the ER, the redox conditions and the pH in the periplasm of *E. coli* are likely to be similar to the conditions in the surrounding medium and thus may vary strongly with growth conditions. Therefore, the pH dependence of the DsbA/glutathione equilibrium may indeed be important in vivo. Very little is known about the thiol-dependent redox state of the periplasm of *E. coli*. In principle, there may be stationary thiol reagents in the periplasm provided by proteins as well as thiols (for instance peptides) that freely diffuse from the medium into the periplasmic space. Regarding the intestinal lumen as a natural environment of *E. coli*, where the growth conditions are anaerobic, it also may be important to discriminate between the oxygen- and the thiol-dependent redox potential, because the absence of oxygen does not necessarily exclude the appearance of disulfide components in the intestinal lumen. Although the *dsbA* gene has been shown not to be essential for bacterial growth under aerobic conditions (Bardwell et al., 1991), it may have an important function in allowing periplasmic disulfide bridges to form under anaerobic conditions due to its intrinsic oxidizing redox properties. The extent and necessity of protein disulfide bond formation in bacteria living under anaerobic conditions is presently unknown and and remains to be elucidated.

Materials and methods

Materials

DTNB, DTT_{ox}, GSH, and GSSG were from Sigma Chemical Co. (St. Louis, Missouri). DTT was purchased from Biomol Feinchemikalien GmbH (Hamburg) and GdmCl from Schwarz/Mann (Orangeburg, New York). All other chemicals were from Merck (Darmstadt) and of the highest purity available. DE52 cellulose was obtained from Whatman BioSystems and phenyl superose (2 mL bed volume) was purchased from Pharmacia LKB Biotechnology Inc. Oligonucleotides were synthesized with an Applied Biosystems 380A synthesizer using the phosphoramidite method. The plasmid pASK40 was a generous gift of Dr. A. Skerra (Max-Planck-Institut für Biophysik, Frankfurt, Germany).

Methods

General methods

Molecular cloning techniques were based on Sambrook et al. (1989). SDS-polyacrylamide gel electrophoresis was performed according to Fling and Gregerson (1986) using 15% (w/v) acrylamide gels.

Cloning, expression, and purification of DsbA

The gene coding for DsbA was amplified from the genome of the *E. coli* K12 wild-type strain W3110 (Bachmann, 1972) by the polymerase chain reaction using oligonucleotide primers based on the published *dsbA* sequence (Bardwell et al., 1991). The amplified gene containing the natural ribosomal binding site and the natural signal sequence was cloned into a derivative of the expression vector pASK40 (Skerra et al., 1991) via the restriction sites HindIII and BamHI. Cells of *E. coli* JM83 (Yanisch-Perron et al., 1985) harboring the DsbA expression plasmid were grown in 2 L LB-medium containing ampicillin (100 μg/mL) at 26 °C. At an optical density at 550 nm of 1.0, IPTG was added to a final concentration

of 1 mM, and the cells were grown further for 16 h. The cells were harvested by centrifugation (4,000 × *g*, 4 °C, 20 min), suspended in 20 mL ice-cold BBS/EDTA buffer (200 mM boric acid/NaOH, pH 8.0, 160 mM NaCl, 5 mM EDTA), and stirred on ice for 1 h. The suspension containing the spheroplasts was centrifuged (48,000 × *g*, 30 min, 4 °C), and the supernatant was dialyzed against 5 L of buffer A (10 mM MOPS/NaOH, pH 7.0) and was applied onto a DE52 column (10 × 1.5 cm) equilibrated with buffer A. After washing the column with the same buffer, DsbA was eluted with 500 mL of a linear gradient of 0–500 mM NaCl in buffer A, and fractions of 5 mL were collected. Fractions 9–15 containing DsbA (corresponding to NaCl concentrations of 45–75 mM) were combined, dialyzed against 2 × 2 L buffer B (1.0 M ammonium sulfate, 100 mM NaCl, 20 mM Tris/HCl, pH 8.0), and applied onto a phenyl superose FPLC column (2 mL, Pharmacia). After washing the column with buffer B, DsbA was eluted as a single peak using a linear gradient (60 mL) of 1.0–0 M ammonium sulfate in buffer B. Fractions containing DsbA were combined and dialyzed against distilled water. Typically, 15 mg of purified, oxidized DsbA were obtained by this procedure.

Protein concentration

The molar extinction coefficient of unfolded, oxidized DsbA (ε_{280nm} = 21,740 M^{-1} cm^{-1}) was calculated from its amino acid sequence as described by Gill and von Hippel (1989). From the comparison of the absorbance of native and denatured DsbA (6 M GdmCl) at 280 nm, an absorption coefficient of $A_{280nm,1mg/mL,1cm}$ = 1.10 was determined for the native, oxidized protein. Protein absorption spectra were recorded on a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer.

Analysis of free thiol groups in the DsbA preparations by Ellman's reagent (DTNB) was carried out at protein concentrations of 50 μM and DTNB concentrations of 0.3 mM in 80 mM sodium phosphate, pH 8.0, 1 mM EDTA, 2% (w/v) SDS. After incubation for 15 min at room temperature, the absorbance at 412 nm was recorded (ε_{412nm} = 13,600 M^{-1} cm^{-1} per free thiol; Ellman, 1959).

The purity of the DsbA preparations was estimated from densitometric scans of 15% SDS-polyacrylamide gels stained with Coomassie blue using an Elscript 400 densitometer (Hirschmann).

Fluorescence measurements

All fluorescence experiments were performed at 30 °C on a Perkin-Elmer MPF-3L spectrometer. All buffers used contained EDTA (1 mM) and were filtered (pore size 0.2 μm). To exclude air oxidation, the solutions were degassed and subsequently flushed with nitrogen. Equilibration of DsbA at different GSH/GSSG ratios (except for incubation times ≤20 min at pH 8.5) was performed in an anaerobic chamber under nitrogen atmosphere. Samples were removed immediately before the fluorimetric measurements. The stability of the thiol titers was verified by determination of free sulfhydryls according to Ellman (1959). Protein concentrations ranged from 0.4 to 2.0 μM. The pH dependence of the DsbA emission spectra and the DsbA/glutathione equilibria were measured in the following buffers each containing 1 mM EDTA: pH 4.0, 100 mM formic acid/NaOH; pH 5.0, 100 mM acetic acid/NaOH; pH 6.0–7.0, 100 mM sodium phosphate; pH 8.0–8.5, 100 mM Tris/HCl. The DsbA fluorescence was shown to be independent of the buffers used. For spectra of reduced DsbA, DTT was added to a final concentration of 10 mM. The fluorescence intensities were measured 30 min after addition of DTT and corrected for the volume increase. It was verified that no further fluorescence changes occurred after 30 min of incubation.

To study the redox equilibrium of DsbA with DTT and glutathione, the change in fluorescence intensity at 322 nm was recorded (excitation wavelength: 280 nm), where the difference between the emission of oxidized and reduced DsbA is maximal. Fluorescence titration experiments with DTT were carried out in 100 mM sodium phosphate, pH 7.0, 1 mM EDTA. Twenty-microliter portions of a DTT solution (10 μM) were added stepwise to a solution of oxidized DsbA (0.84 μM, 2 mL). Ten minutes after each addition, the fluorescence intensity of the sample was recorded for 1 min, averaged, and corrected for the volume increase. It was verified that no further fluorescence change could be detected after a 10-min period. Titration experiments with DTT in the presence of 1 mM DTT_{ox} were performed under identical conditions, except that a protein concentration of 0.42 μM was used.

For determination of the equilibrium constants of the DsbA/glutathione redox system in the range from pH 4 to 8.5, oxidized DsbA (0.42 μM) was incubated at 30 °C in the presence of 0.1 mM GSSG and different concentrations of GSH (0–2,000 μM). Incubation times necessary to reach the equilibrium were dependent on pH and ranged from 20 min at pH 8.5 to 72 h at pH 4. The equilibrium concentrations of GSH and GSSG were calculated according to Equations 9–11, where $[GSH_0]$ and $[GSSG_0]$ are the initial concentrations of GSH and GSSG, R is the relative amount of reduced DsbA at equilibrium, $[PDI_0]$ is the initial concentration of DsbA (oxidized form), F is the measured fluorescence intensity, and F_{ox} and F_{red} are the fluorescence intensities of completely oxidized and reduced DsbA, respectively. The data were fitted according to Equation 5. In all experiments, correlation coefficients ≥0.995 were obtained after nonlinear regression.

$$[GSH] = [GSH_0] - 2R[PDI_0] \quad (9)$$

$$[GSSG] = [GSSG_0] + R[PDI_0] \quad (10)$$

$$R = (F - F_{ox})/(F_{red} - F_{ox}). \quad (11)$$

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