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Why Is DsbA Such an Oxidizing Disulfide Catalyst?

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Summary

DsbA, a member of the thioredoxin family of disulfide oxidoreductases, acts in catalyzing disulfide bond formation by donating its disulfide to newly translocated proteins. We have found that the two central residues within the active site Cys-30-Pro-31-His-32-Cys-33 motif are critical in determining the exceptional oxidizing power of DsbA. Mutations that change these two residues can alter the equilibrium oxidation potential of DsbA by more than 1000-fold. A quantitative explanation for the very high redox potential of DsbA was found by measuring the pK_a of a single residue, Cys-30. The pK_a of Cys-30 varied dramatically from mutant to mutant and could accurately predict the oxidizing power of each DsbA mutant protein.

Introduction

Disulfide bond formation, an essential step in the folding pathway of many proteins, is a catalyzed reaction within both eukaryotes and prokaryotes (Goldberger et al., 1963; Bardwell et al., 1991; Freedman, 1994). The active site of the DsbA protein, a disulfide catalyst, is itself a disulfide bond that is transferred catalytically to folding proteins in the periplasm of Escherichia coli, resulting in their oxidation (Wunderlich and Glockshuber, 1993a; Wunderlich et al., 1993; Zapun et al., 1993; Bardwell, 1994; Zapun and Creighton, 1994). The sequence motif Cys-30–Pro-31–His-32–Cys-33 constitutes the active site of E. coli DsbA. The disulfide bond that forms between these two cysteines is regenerated in an oxidative process that requires the integral membrane protein DsbB (Bardwell et al., 1993; Kishigami et al., 1995; Guilhot et al., 1995). Two additional proteins, DsbC (a periplasmic protein) and DsbD (an inner membrane protein), may be involved in catalyzing intramolecular disulfide isomerization and reduction reactions, respectively (Zapun et al., 1995; Missiakas et al., 1995).

The equilibrium constant (K_{ox}) for forming disulfides by exchange with glutathione redox buffer is a measure commonly used to compare the oxidizing power of different proteins (Gilbert, 1990). The K_{ox} value for the active site disulfide of DsbA is about 0.1 mM, making it a very potent donor of disulfide bonds (Wunderlich and Glockshuber, 1993a; Zapun et al., 1993). For comparison, thioredoxin, which is thought to function as a dithiol reductant in the cytoplasm, has a K_{ox} value of about 2 M at pH 7.5 (Holmgren, 1985). Since oxidation is the primary function of DsbA in its role as a protein-folding catalyst, the answer to the question of why DsbA is so oxidizing is crucial (Zapun and Creighton, 1994). Two prior observations shed light on this question. First, the pK_a of the most N-terminal of the two cysteines in the active site of DsbA (Cys-30) is extremely low, suggesting that DsbA is very prone to leave its disulfide behind in thiol disulfide exchange reactions (Nelson and Creighton, 1994). Second, oxidized DsbA is less stable than reduced DsbA (Wunderlich et al., 1993; Zapun et al., 1993), providing a thermodynamic drive to aid in the transfer of disulfides from DsbA to folding proteins. Despite the solution of a high resolution crystal structure for DsbA (Martin et al., 1993a) and extensive biochemical experimentation (Zapun et al., 1993), the basis for the exceptional oxidizing power of DsbA remains puzzling. However, there is good evidence that the two residues found in between the active site cysteines play a role in determining the redox potential of DsbA and other oxidoreductases within the thioredoxin superfamily. Each subfamily possesses a characteristic dipeptide sequence within the active site: for DsbA, it is PH; for thioredoxin, it is GP; for eukaryotic protein disulfide isomerase, it is GH (Martin, 1995). If these residues are substituted for each other, the redox potential of the mutant protein is shifted in the direction of the protein from which the dipeptide sequence originated (Lundström et al., 1992; Wunderlich, 1994). Electrostatic calculations suggest that His-32, among others, should help stabilize the thiolate ion of Cys-30 and thus favor the reduced form of DsbA (Gane et al., 1995). We have found that the two central residues in the CXXC motif of DsbA modulate the redox potential of DsbA by strongly affecting both the pK_a of Cys-30 and the relative stabilities of the oxidized and reduced forms. These results allow us to propose a model explaining the oxidizing power of DsbA.

Results

To understand the importance of the CXXC motif in oxidoreductases, site-directed mutagenesis was employed to exchange randomly the two X residues separating the active site cysteines in the disulfide catalyst DsbA. We then analyzed the oxidizing properties of the resulting mutant proteins using both in vivo screens and in vitro biophysical techniques. *dsbA* mutants were classified using a modification of the selection that was originally used to discover the *dsbA* and *dsbB* genes (Bardwell et al., 1991, 1993, 1994). This selection is based on the disulfide indicator properties of the MalF–β-galactosidase 102 fusion protein.

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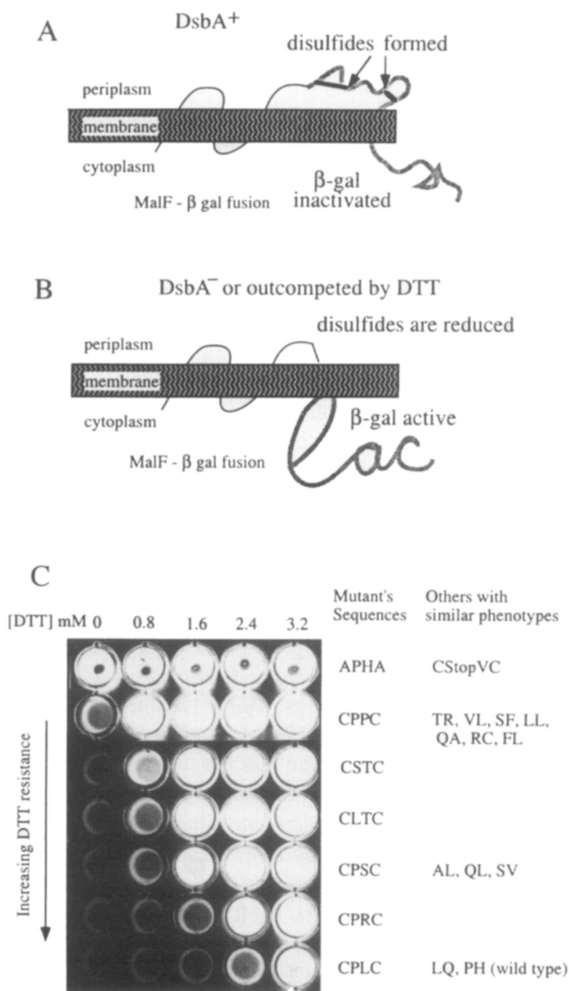


Figure 1. In Vivo Test for Function of the Disulfide Catalyst DsbA Using a Disulfide Indicator Protein

(A) The MalF–β-galactosidase 102 fusion protein is inactivated in $DsbA^+$ strains when disulfides are formed in the portion of β-galactosidase that is exported to the periplasm.

(B) The presence of the null mutation in *dsbA* or addition of sufficient DTT to the media to overcome the oxidizing influence of DsbA will reduce the disulfides in fusion protein, resulting in a Lac^+ phenotype of this disulfide indicator protein.

(C) Active site mutations in *dsbA* classified according to the level of DTT that is required to cause the strain to become Lac^+. Microtiter dish wells containing minimal media supplemented with various concentrations of DTT were inoculated with strains that contained both the MalF–β-galactosidease 102 fusion and various *dsbA* active site mutants carried on derivatives of the plasmid pUG1. β-Galactosidase activity was then detected with the fluorescent β-galactosidase substrate 4-methylumbelliferyl-β-D-galactoside. The predicted amino acid sequence of the active site CXXC motif, corresponding to amino acids 30–33 in wild-type DsbA, are shown. Only representative mutants are illustrated.

This protein has substantial β-galactosidase activity if the strain in which this fusion protein is expressed contains a null mutation in the *dsbA* or *dsbB* genes. However, in strains that are wild type for the *dsb* genes, the fusion has virtually no β-galactosidase activity. The DsbA protein is apparently able to oxidize, and thus inactivate, the portion of this fusion protein that is exposed to the periplasm (Figures 1A and 1B). We have developed a semiquantitative test for DsbA function by exploiting the observation that *dsbA* mutants vary in their ability to overcome millimolar levels of dithiothreitol (DTT) added to the growth media. In the absence of DTT, all active site mutants were Lac^-, indicating that they had at least some DsbA activity. However, addition of DTT to the growth media caused the strains to exhibit β-galactosidase activity. The mutants were ranked based on their ability to show β-galactosidase activity at DTT concentrations ranging from 0 to 3.2 mM. The mutants varied from showing near wild-type activity to activities only slightly above the null mutant background (Figure 1C).

DsbA protein from nine representative mutants was then purified, and the relative oxidizing power of these mutant proteins was determined using glutathione as a standard. The glutathione redox scale compares the ability of proteins to transfer their disulfides to reduced glutathione (Gilbert, 1990). This measure is simply the equilibrium constant (K_{ox}) for the following reaction:

$$DsbA_{red} + GSSG \rightleftarrows DsbA_{ox} + 2\,GSH \quad (1)$$

$$K_{ox} = \frac{[DsbA_{ox}][GSH]^2}{[DsbA_{red}][GSSG]}. \quad (2)$$

The smaller the equilibrium constant K_{ox}, the more the oxidation of glutathione is favored. Strongly oxidizing disulfides such as the one present in the active site of DsbA have small equilibrium constants. The K_{ox} of DsbA is about 0.1 mM (Wunderlich and Glockshuber, 1993a; Zapun et al., 1993), making it one of the most oxidizing protein disul-

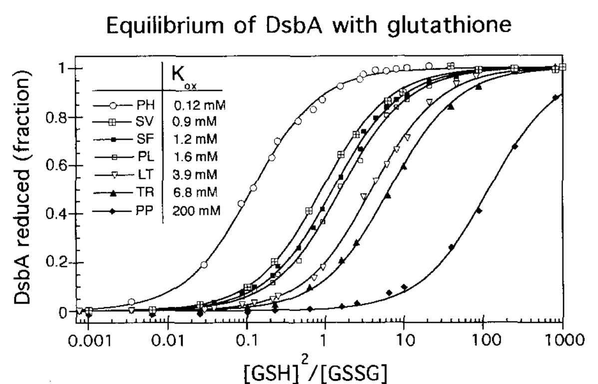


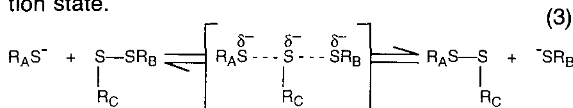
Figure 2. Redox Equilibrium of DsbA Active Site Mutants with Glutathione

The proportion of DsbA present in the reduced form at equilibrium with various mixtures of reduced (GSH) and oxidized (GSSG) glutathione was measured by exploiting the 3.2-fold greater fluorescence of reduced as compared with oxidized DsbA, and the equilibrium concentrations of oxidized and reduced DsbA and GSH and GSSG were calculated as described in the Experimental Procedures. The equilibrium constants (K_{ox}) for the mutants were determined as described by Wunderlich and Glockshuber (1993b). Owing to their very similar K_{ox} values, the data sets from mutants ST (K_{ox} = 1.27 mM), PS (K_{ox} = 6.1 mM), and QL (K_{ox} = 6.4 mM) were almost perfectly superimposable with those from SF (K_{ox} = 1.24 mM) and TR (K_{ox} = 6.8 mM) and are consequently not shown.

fides known. These redox equilibrium constants of our DsbA mutant proteins were measured by a very convenient method that exploits quenching of tryptophan fluorescence by the active site disulfide bond (Wunderlich and Glockshuber, 1993a). The redox equilibrium constants measured span a wide range from 0.1 mM for wild-type DsbA to about 200 mM for a mutant with the sequence CPPC (Figure 2). The wide range of redox potentials shows that the two central residues are critical in determining the redox properties of DsbA. The most reducing mutant that we have found has not only the sequence of Arabidopsis thioredoxin (CPPC) (Rivera-Madrid et al., 1995) but also a redox potential that approaches that of E. coli thioredoxin, which has been measured at about 2 M (Holmgren, 1985). This shows that one can change DsbA from a very oxidizing protein to one with a thioredoxin-like redox potential simply by changing the active site sequence to reflect that of a thioredoxin. A very similar result was found by Wunderlich (1994) who generated a DsbA active site mutant with the sequence CGPC, which is commonly found in thioredoxins, and found its K_{ox} to be 1 M.

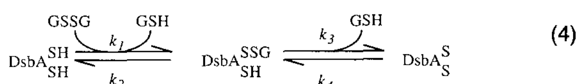
Thiols are only reactive in disulfide exchange reactions when they are deprotonated to the thiolate ion. The generalized disulfide interchange reaction diagrammed in equation 3 is a concerted reaction going through a single transition state.

(3)



It is important to realize that the reaction is completely symmetrical and that the identity of the reaction partners (R_AS, R_BS, and R_CS) allows one to define the sulfhydryls involved in any specific reaction. The pK_a of thiol groups determines not only their intrinsic reactivity but also their reactivity when present within a disulfide bond. Szajewski and Whitesides (1980) have shown that for small molecules the driving force in determination of the equilibrium constant for this reaction is the relative pK_a of the SH groups. Thus, if R_AS^- has a lower pK_a than R_BS^-, the negative charge will be better stabilized on R_AS^- than on R_BS^- and the reaction will shifted to the left. In the case of DsbA, equation 3 applies to each of the two part-reactions:

(4)



in which $K_I = k_1/k_2$; $K_{II} = k_3/k_4$; $K_{ox} = K_I K_{II}$. The sulfide of Cys-30 is depicted in superscript as $DsbA^{SH}$, and the sulfide of Cys-33 is depicted in subscript as $DsbA_{SH}$. $DsbA^{SSG}_{SH}$ is the mixed disulfide with glutathione, and $DsbA^{S}_{S}$ indicates the oxidized form of the enzyme. Sulfhydryls are designated SH for reasons of simplicity. The important player in the exchange reaction (depicted in equation 4) is Cys-30. It has been shown that the mixed-disulfide intermediate is formed between this residue and the substrate (Zapun et al., 1994), and in the wild-type enzyme, this thiol has a pK_a of around 3.5 (Nelson and Creighton, 1994). In the reaction scheme shown in equation 4, the equilibrium constant K_{II} will not be strongly influenced by the pK_a of Cys-30, since this residue plays the role of R_C. The rates of formation of the inter- and intramolecular disulfide bonds are thus affected approximately to the same extent by the pK_a of Cys-30 (Nelson and Creighton, 1994). The reaction of the mixed disulfide with GSH (*k2*), on the other hand, is favored because of the stabilization of the negative charge on Cys-30. Thus, in wild-type DsbA, Cys-30 is a superb leaving group. This drives the reaction to the left, making DsbA a highly oxidizing disulfide catalyst (Nelson and Creighton, 1994).

Since pK_a values are affected by nearby electrostatic forces, we reasoned that by mutating the residues adjacent to the active site cysteines we might have affected the pK_a of Cys-30. The pK_a of Cys-30 can be determined on the reduced enzyme by titration of the absorption at 240 nm against pH, since the thiolate ion has a lower absorption at this wavelength than the thiol group (Nelson and Creighton, 1994). Indeed, the pK_a of Cys-30 was found to be dramatically affected in our mutants (Figure 3). The most reducing mutant, CPPC, had a measured pK_a of approximately 6.7, which is over 3 pH units higher than that of wild type. Furthermore, the pK_a was found to vary in proportion to K_{ox}, suggesting that pK_a plays a very important role in determining K_{ox} (Figure 4). Thus, we have

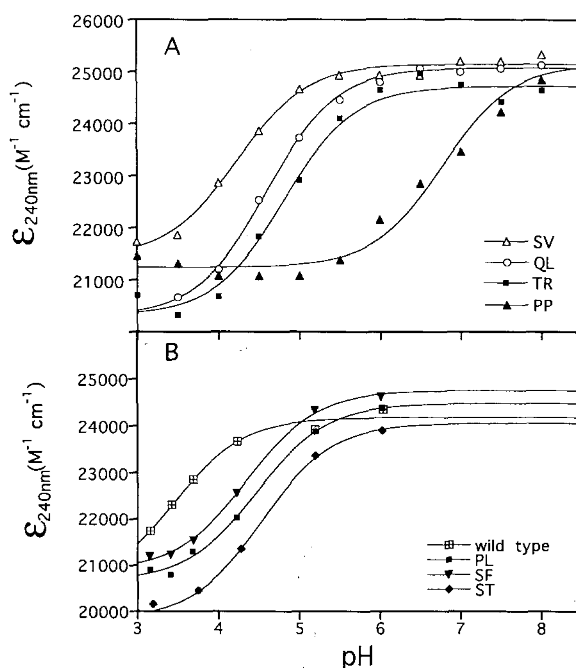


Figure 3. Determination of the pK_a Values of the Active Site Cysteine, Cys-30, in DsbA Mutants

The higher specific absorption of the thiolate ion at 240 nm relative to the unionized thiol group was used to determine the pK_a of Cys-30 by monitoring UV absorption during pH titration. The absorption properties specific to reduced DsbA mutant proteins are shown as a function of pH. ε_{240} is the extinction at 240 nm. The pK_as of Cys-30 shown in Table 1 were calculated by fitting the data points according to equation 6. (A) shows mutants SV, QL, TR, and PP, while (B) shows wild type and mutants PL, SF, and ST.

Table 1. pK_a Values of Cys-30 in DsbA Mutants

Mutant	pK_a
PH (wild type)	3.42 ± 0.18
SV	4.23 ± 0.05
SF	4.34 ± 0.05
PL	4.42 ± 0.10
ST	4.45 ± 0.06
QL	4.59 ± 0.03
TR	4.76 ± 0.05
LT	4.86 ± 0.03
PP	6.73 ± 0.12

found that the two central residues within the active site play a critical role in determining both the oxidative power of DsbA and the pK_a of Cys-30. We now wished to explore the exciting possibility that the redox equilibrium potential of DsbA is determined entirely by the pK_a of Cys-30. Szajewsky and Whitesides (1980) showed for small molecule disulfides that if one knows the pK_a of all sulfhydryls involved in a disulfide exchange reaction, one can calculate the equilibrium constant of the reaction. The pK_a of glutathione is known to be 8.7 (Szajewski and Whitesides, 1980). The pK_a of Cys-33 is only known within limits. It is 9.5 in the absence of the first cysteine. Its pK_a is apparently increased to above 10 by the low pK_a of the first cysteine (Nelson and Creighton, 1994). We can now calculate, using the Szajewski and Whitesides equation (see equation 7), what the K_{ox} value of the mutant proteins would be if it were determined entirely by the pK_a values of the involved sulfides. We used the known pK_a of glutathione (8.7), varied the pK_a of Cys-30 between 3 and 7 to simulate the effects of the mutants, and used two outside limits (9.5 and 14) for the pK_a of Cys-33. The relationship of these predicted equilibrium constants to the pK_a of the first cysteine is shown as two dashed lines in Figure 4: the upper line is calculated using the upper limit of 14 for the pK_a of Cys-33, while the lower one is calculated using the lower limit of 9.5. The fact that these two lines are close together indicates that the predicted equilibrium constants are rather insensitive to the pK_a of Cys-33. This is because cysteines with high pK_a values are in the protonated (unreactive) form at the pH used, resulting in only a slight dependence of K_{ox} on thiol pK_a (Gilbert, 1990). In contrast, the steep slope of these lines indicates that the pK_a of Cys-30 is very important in determining the equilibrium redox constants. The equilibrium constants actually measured for the DsbA mutant proteins (Figure 4, solid line) are very similar in value to those predicted by the Szajewski and Whitesides formulation (Figure 4, dashed lines) and vary strongly with pK_a in almost exactly the same way as predicted. The measured equilibrium constants are consistent with the pK_a of Cys-33 lying between 9.5 and 14. That the redox equilibrium constant of the entire DsbA protein can be predicted simply from the pK_a value of a single residue, Cys-30, implies that this pK_a is the dominant factor controlling the oxidizing power of DsbA. Thus, the answer to the question of why wild-type DsbA is so oxidizing appears to reside in the abnormally low pK_a of Cys-30.

A consistent thermodynamic cycle has also been established that links the redox properties of the DsbA protein to the relative stabilities of oxidized and reduced DsbA (Zapun et al., 1993, 1994; Wunderlich and Glockshuber, 1993a; Wunderlich et al., 1993; Wunderlich, 1994). The difference in stability between the oxidized and reduced forms of DsbA protein ($\Delta\Delta G_{fold}$) should be equal to the differences in the stability of the disulfide present in native and denatured DsbA ($\Delta\Delta G_{ss}$). The value of $\Delta\Delta G_{ss}$, in turn, can be calculated from the equilibrium constants for disulfide bond formation in native and denatured DsbA using the following formula:

$$\Delta\Delta G_{ss} = -RT\ln\left(\frac{K_{ox}}{K^{u}_{ss}}\right). \quad (5)$$

The value of K_{ox} has been measured for the active site mutants in DsbA. K^{u}_{ss} is the equilibrium constant of disulfide bond formation in the denatured state. The redox potentials of several CXXC disulfides in short peptides have been measured by Siedler et al. (1993). The values vary only over a small range showing an insensitivity to the sequence of the central residues. Thus, in the denatured state, the redox potential of the mutants is expected to be rather similar to that of the wild-type DsbA, in which K^{u}_{ss} has been determined to be 170 mM (Zapun et al., 1993).

Do our mutants obey the thermodynamic linkage of $\Delta\Delta G_{ss} = \Delta\Delta G_{fold}$? The stability of wild type and five of our mutants was determined by monitoring guanidinium hydrochloride–induced folding transitions using the fluorescence changes that occur upon unfolding of DsbA (Figure 5). The data for four of the five mutants were consistent with highly cooperative, reversible folding transitions and were analyzed accordingly (Table 2). These mutants all showed a decreased difference in stability between the oxidized and reduced forms ($\Delta\Delta G_{fold}$) as expected by the

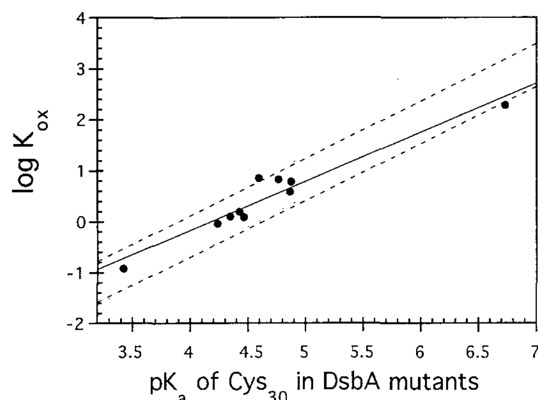


Figure 4. The Thermodynamic Oxidizing Power of DsbA Active Site Mutants Correlated with the pK_a of Cys-30

Experimentally measured values of K_{ox} and pK_a are shown as circles that are fitted with a solid line. The dashed lines show the predicted relationship between $K_{ox,\,pred}$ and the pK_a of Cys-30 using the relationship derived for small molecule disulfides (Szajewski and Whitesides, 1980). The upper dashed line shows the relationship assuming the pK_a of Cys-33 is 14; the lower dashed line shows the relationship if the pK_a of Cys-33 is 9.5.

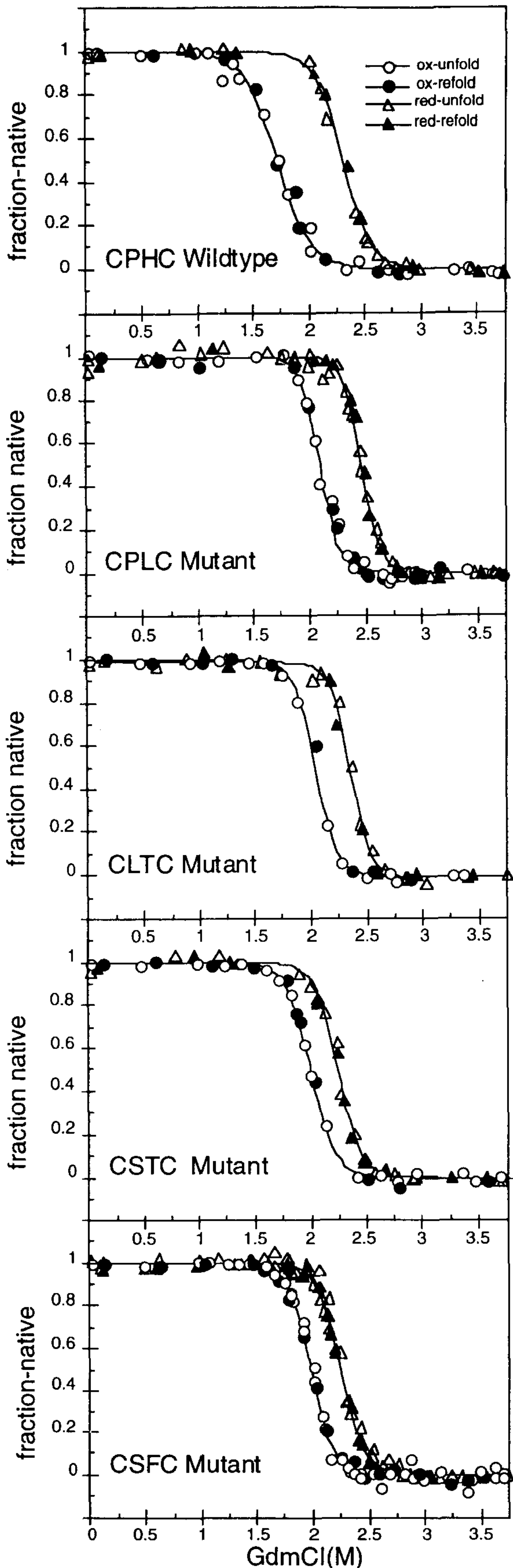


Figure 5. Folding Transitions of the DsbA Mutants

Guanidinium chloride (GdmCl)-dependent unfolding/folding equilibrium of oxidized DsbA (shown as circles) and reduced DsbA (shown as triangles) for wild-type protein and four mutant DsbA proteins that contain mutations in their active site. The fraction of native DsbA molecules was determined by relative fluorescence. The folding transitions and the curve fitting were done as described previously (Wunderlich et al., 1993). Unfolding transitions are shown as open symbols, whereas folding transitions are shown as closed symbols.

linkage. The long extrapolation required to reach zero guanidinium concentration and the small differences in stability between oxidized and reduced proteins make it unlikely that $\Delta\Delta G_{ss}$ and $\Delta\Delta G_{fold}$ will be found to be precisely equal. Nevertheless, for the wild type and the mutants measured, there was good agreement between $\Delta\Delta G_{ss}$ and $\Delta\Delta G_{fold}$ as demanded by the thermodynamic cycle (Table 2). Unfortunately, the oxidized form of the CPPC mutant showed incomplete reversibility and decreased cooperativity, making exact computation of a $\Delta\Delta G_{fold}$ value for this mutant difficult. However, equation 5 can be used to predict that the oxidized form of this mutant will be very slightly more stable than the reduced form, in sharp contrast with wild type and all the other mutants. This was indeed observed both for guanidinium hydrochloride–induced and thermally induced folding transitions (data not shown).

The above results show that the redox equilibrium values can be used to predict the differences in stability between oxidized and reduced DsbA. This is true for wild-type DsbA and for a number of DsbA mutants (Zapun et al., 1993; Wunderlich, 1994). Thus, the redox properties of DsbA can be accounted for either by the observed differences in stabilities or by the pK_a of Cys-30. How these two apparently different explanations actually represent two sides of the same coin is discussed below.

Discussion

DsbA is effective as a catalyst of disulfide bond formation in part because its active site disulfide is so oxidizing. Two unusual properties of DsbA have been observed that may explain why DsbA is so oxidizing: the destabilizing effect of the active site disulfide (Wunderlich et al., 1993; Zapun et al., 1993) and the abnormally low pK_a of Cys-30 (Nelson and Creighton, 1994). Our results suggest a model for the oxidizing power of DsbA that combines both of these explanations. We have found that both the low pK_a of Cys-30 and the difference in stability between the oxidized and reduced states of the enzyme appear to be sufficient to account for the redox properties of DsbA quantitatively. The low pK_a of Cys-30 results in it being negatively charged at neutral pH. This makes DsbA highly oxidizing by allowing it to exit disulfide exchange reactions with Cys-30 in the reduced thiolate ion form. The higher stability of the reduced state, in turn, makes the reduced form of DsbA more energetically favored. Electrostatic calculations suggest that a negative charge on Cys-30 may be involved in stabilizing interactions with His-32 and several other residues (Gane et al., 1995). A direct role of His-32 in stabilizing interactions is also suggested both by its

Table 2. Folding Transitions

	Transition Midpoint (M GdmCl)	Cooperativity (kcal/mol·M GdmCl)	ΔG_{fold}[a] (kcal/mol)	$\Delta\Delta G_{fold}$[b] (kcal/mol)	$\Delta\Delta G_{ss}$[c] (kcal/mol)
Wild Type				4.1	4.3
Oxidized	1.71	4.1 ± 0.4	−7.0 ± 0.7		
Reduced	2.29	4.9 ± 0.2	−11.1 ± 0.3		
CSFC				2.8	2.9
Oxidized	1.97	6.0 ± 0.2	−11.5 ± 1		
Reduced	2.24	5.6 ± 0.2	−14.3 ± 1.5		
CSTC				2.6	3.0
Oxidized	1.99	5.6 ± 0.2	−11.7 ± 1		
Reduced	2.24	5.9 ± 0.4	−14.3 ± 1.5		
CPLC				3.4	2.8
Oxidized	2.09	5.3 ± 0.4	−12.3 ± 1.1		
Reduced	2.46	7.0 ± 0.5	−15.7 ± 1.7		
CLTC				3.1	2.3
Oxidized	2.04	6.6 ± 0.6	−12.0 ± 1.1		
Reduced	2.36	6.9 ± 0.6	−15.0 ± 1.6		

[a] ΔG_{fold} = transition midpoint × averaged cooperativity.
[b] $\Delta\Delta G_{fold} = \Delta G_{fold,ox} - \Delta G_{fold,red}$.
[c] $\Delta\Delta G_{ss} = -RT\ln\left(\frac{K_{ox}}{K_{ss}^{u}}\right)$

proximity to the ionized Cys-30 and by the changes in stability that accompany its mutation. These interactions stabilize both the thiolate ion and the folded state of the reduced protein. The absence of the thiolate ion in the oxidized form causes it to be less stable.

Although it appears that the difference in stability between the oxidized and reduced forms is caused by stabilizing interactions present in the reduced form (Nelson and Creighton, 1994; Gane et al., 1995), it could in principle be caused by additional tension present in the oxidized form (Wunderlich et al., 1993; Wunderlich, 1994). It is remarkable that the pK_a values alone are sufficient to predict quantitatively the redox properties of wild-type DsbA and a large number active site mutants, since it suggests that this property in the enzymes is governed by chemistry that is no more complicated than that taking place in small molecules. It also means that it is not necessary to postulate that tension present in the oxidized form of DsbA is responsible for its oxidizing power. Although there is a formal possibility that the mutations affect the strain of the disulfide bond, this tension model is disfavored by several previous observations. In the three-dimensional structure of DsbA, there is no evidence for strain in the active site disulfide, all bond angles are close to optimal, and the α carbons of the active site can be exactly superimposed upon the structure of the active site of thioredoxin, even though thioredoxin is much less oxidizing than DsbA (Martin et al., 1993a; Martin, 1995). In addition, a mutant in which Cys-33 is replaced with serine is destabilized 1.5 kcal per molecule upon formation of a mixed disulfide with glutathione (Zapun et al., 1994). No evidence of noncovalent interactions of glutathione with DsbA are apparent from their interaction kinetics (Zapun et al., 1994). It is reasonable to assume that this mixed disulfide, by eliminating the thiolate ion of Cys-30, is eliminating the stabilizing interactions that would normally take place in reduced DsbA between this thiolate and other residues.

Electrostatic calculations comparing the structure of thioredoxin and DsbA point to the importance of the His-32 of DsbA in explaining the big difference in redox potential between DsbA and thioredoxin (Gane et al., 1995). Mutational studies are underway to test the electrostatic predictions that other residues, including Glu-37, Glu-38, and Gln-97, are also important in determining the redox potential of DsbA.

The DsbA mutants that we have generated may prove useful in down-regulating the extremely oxidizing environment of the periplasm for biotechnological applications. Comparison between the folding of eukaryotic protease inhibitors expressed in the periplasm of E. coli with folding of endogeneous periplasmic proteins suggests that the oxidizing environment provided by wild-type DsbA may be too strong for the proper folding of eukaryotic proteins (Ostermeier and Georgiou, 1994; Walker and Gilbert, 1994; Wunderlich and Glockshuber, 1993b). The effectiveness of DsbA as an in vivo catalyst of disulfide bond formation depends both on how strongly oxidizing it is as measured by its equilibrium redox potential, a thermodynamic property, and upon the kinetics at which it participates in disulfide interchange reactions. The equilibrium redox potential can be easily measured, but the kinetic behavior of mutant proteins is more difficult to determine, providing an important role for in vivo genetic screens. The ranking of the in vivo phenotype of the mutant did not exactly follow the ranking by equilibrium redox potential. This suggests that the mutants are kinetically affected in ways that do not exactly parallel their equilibrium redox properties. One potentially useful class of mutants, which includes the mutant PL, are phenotypically similar to wild type in vivo, implying that they are kinetically still capable

of extremely fast disulfide interchange reactions but encode mutant proteins that are substantially less oxidizing than wild type.

Although the overall equilibrium constants are predicted well by the Szajewski and Whitesides formula, the individual rate constants for the reaction of DsbA with glutathione are in all cases faster than predicted (Nelson and Creighton, 1994; data not shown). These rate enhancements, of up to 6000-fold, are good evidence that DsbA is acting as a catalyst in disulfide exchange reactions with glutathione. DsbA is capable of even more rapid disulfide exchange reactions with proteins (Wunderlich and Glockshuber, 1993b; Zapun and Creighton, 1994). These fast rates are consistent with its in vivo function to rapidly transfer its disulfide bond to proteins. Although the factors determining the oxidizing redox potential of DsbA are now becoming clear, the mechanism of its rapid kinetics of interaction with folding proteins and the means by which DsbB effects its reoxidation remain somewhat mysterious.

Experimental Procedures

Mutagenesis of DsbA

The plasmid pUG1 was constructed to bring the Ala-30/Ala-33 null mutant of the *dsbA* gene under the inducible *trc* promoter. This plasmid was constructed by ligating a 2374 bp BssHII–AlwNI fragment from pRBI-PDI-CC30/33AA (Wunderlich et al., 1995) with a 2906 bp fragment from pBJ41 (Bardwell et al., 1991). This mutant has both active site cysteines mutated to alanine and is inactive both in vitro and in vivo. A control plasmid pJW1717 that contained the wild-type sequence at the active site of DsbA but was otherwise identical to pUG1 was constructed in the same manner from the wild-type pRBI-PDI plasmid and pBJ41. The low level of DsbA expressed from the uninduced *trc* promoter in pJW1717 was sufficient to complement the *dsbA* null mutant *dsbA::kan1*. The active site of the DsbA encoded by pUG1 was then changed from APHA to CNNC, in which N is any amino acid. A null mutant of DsbA was used in the starting plasmid to make the unmutagenized background DsbA⁻. Site-directed mutagenesis of DsbA was carried out using the Sculptor II kit (Pharmacia) using single-stranded DNA made from the plasmid pUG1 and the oligonucleotide 5′-CAGAAATATGCAGAACTTCTTCAAACTGATAACANNNNNNGC AGAAGAAAGAGAAAAAC-3′, which was a gift of R. Glockshuber.

Phenotypic Characterization of DsbA Mutants

The randomly mutagenized pUG1 plasmid was introduced into the strain THZ7 by electroporation. THZ7 contains the *dsbA* null mutant *dsbA::kan1*, the MalF–β-galactosidase 102 fusion protein, and the *recA::cat* mutation. THZ7 was constructed by transducing the *recA::cat* mutation into JCB817 using P1vir. JCB817 is MC1000 *phoR zih12::Tn10 dsbA::kan1* (λ*malF–lacZ102*). The *recA* mutation stabilized the λ phage that carried the MalF–β-galactosidase 102 fusion and greatly reduced recombination between mutant alleles present on plasmids and the chromosomal genes.

Strains were screened using two phenotypes that are dependent upon the presence of active DsbA protein: motility and the Lac⁻ phenotype of the MalF–β-galactosidase 102 fusion protein (Bardwell et al., 1991, 1993; Dailey and Berg, 1993). Those *dsbA* mutant plasmids that conferred a DsbA⁺ phenotype upon the strain THZ7 were picked for further analysis. LB agar supplemented with 60 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galacotpyranoside (X-Gal) and 0.4% maltose was used for initial screening of Lac phenotype. Motility plates that contained LB media and 0.4% agar were used for motility screening. The mutants were then classified according to their ability to overcome DTT-mediated reduction of the MalF–β-galactosidase 102 fusion. We filled 24-well tissue culture plates (Flow Laboratories) with 1 ml of M63 media (Bardwell et al., 1991) that, in addition, contained all amino acids except cysteine at 50 μg/ml, 200 μg/ml ampicillin, 60 μg/ml (X-Gal), 0.4% agar, and 0.4% maltose. The DTT content varied in 0.8 mM steps from 0 to 3.2 mM final concentration. Dense liquid cultures of the various *dsbA* mutant strains were inoculated onto the tissue culture plates using sterile toothpicks. The plates were incubated at 37°C for 48 hr, and ranking was determined either according to X-Gal coloring or using an overlay of 0.1 ml of 4-methylumbelliferyl-β-D-galactoside (250 μg/ml in DMSO). Cleavage of this fluorescent analog of β-galactosidase was allowed to proceed for 24 hr at room temperature, and plates were photographed under UV light. The mutants were sorted into five categories according to the minimal DTT concentration that caused the strains to become Lac⁺. Approximate ranking within each category was by intensity of fluorescence. In three experiments done using a total of 36 mutant strains, the ranking of any one mutant varied by an average of two positions. DNA was purified from the mutant derivatives of pUG1 using Qiagen columns, and the *dsbA* gene was sequenced using Sequenase.

DsbA Overproduction and Purification

DsbA mutant proteins were purified from periplasmic extracts essentially as described by Martin et al. (1993b). The resulting DsbA was >95% pure, as judged by densitometric scanning of SDS–polyacrylamide gels, and >98% oxidized, as judged by assay using Ellman's reagent (Riddles et al., 1983). DsbA protein concentrations were determined using an absorption coefficient of 1.10 at 280 nm (A_{280}, 1 cm, 0.1%).

Determination of Redox Equilibrium with Glutathione and the pK_a Values of Cys-30

The redox equilibrium measurements of DsbA were performed as described by Wunderlich and Glockshuber (1993a).

The thiolate ion has a higher absorption at 240 nm than the unionized thiol group, allowing the determination of thiol pK_a by monitoring UV absorption during pH titration (Nelson and Creighton, 1994). The pH of a DsbA solution (0.1–0.2 mg/ml) buffered with a poly buffer that contained 1 mM sodium citrate, borate, and phosphate was adjusted to various pH values ranging from 3 to 8.5 by adding solutions that contained different ratios of 10 mM KOH and HCl. Since many other groups in proteins, including the peptide bond, absorb at 240 nm, it is necessary to compare carefully the absorption at any one pH to the absorption of the same protein in which the thiol group is absent. We determined the absorption specific to reduced form of the proteins by subtracting the pH-dependent absorption of the oxidized form of each of the mutant proteins. These values indicated the amounts of the thiolate ion depending upon pH. The protein concentrations of the solutions were determined by absorption at 280 nm. The pH-dependent absorption was fitted according to the Henderson–Hasselbalch equation

$$A_{Exp} = A_{SH} + \frac{A_{S^-} - A_{SH}}{1 + 10^{(pK_a - pH)}} \quad (6)$$

in which A_{Exp} indicates A_{240}/A_{280} for the experimentally determined value, A_{SH} is A_{240}/A_{280} for the fully protonated form and A_{S^-} is A_{240}/A_{280} for the fully deprotonated form.

The accuracy of this technique is limited at pH extremes. At pH values below 3, DsbA undergoes acid denaturation, as indicated by a time-dependent increase in absorption that occurs as tyrosine residues are exposed to solvent. This phenomena imposes 3 as the lower limit of the usable pH values and decreases the accuracy at which the pK_a of Cys-30 in wild-type DsbA can be determined. At pH values over 7, absorption caused by ionization of tyrosine residues dominates the signal. This decreases the signal to noise ratio and leads to increasing scatter of the data points at high pH values.

Calculation of Rate Constants

The calculations for prediction of the rate constants, k_{pred} (in units of $s^{-1}M^{-1}$), and the overall equilibrium constant, $K_{ox,pred}$ (in units of M), were done according to the formula of Szajewski and Whitesides (1980):

$$\log k = 4.5 + 0.59\, pK_a(A) - 0.4\, pK_a(C) - 0.59\, pK_a(B). \quad (7)$$

Using the pK_a values of the three sulfides involved, we calculated, using equation 7, the predicted rate constant for each of the steps

shown in equation 4. For example, to calculate the rate constant *k3*, Cys-33 is R_A, the Cys-30 is R_C, and GSH is R_B. The four rate constants (*k1*, *k2*, *k3*, and *k4*) were individually calculated and corrected to pH 7, the pH at which the K_{ox} measurements were done, according to Szajewski and Whitesides (1980):

$$\log k_{pred} = \log k - \log [1 + 10^{(pK_a(A) - pH)}], \quad (8)$$

in which log *k* is given from equation 7 and the $pK_a(A)$ is that of reactant R_AS^- of equation 3 corresponding to each of the four rate constants. The overall equilibrium constant K_{ox} was then obtained according to the relation:

$$K_{ox, pred} = (k_{1, pred}k_{3, pred})/(k_{2, pred}k_{4, pred}). \quad (9)$$

Stability Measurements

Guanidinium-induced unfolding and refolding transitions were performed, and the free energy of folding was calculated by extrapolation to zero guanidinium concentration as described by Wunderlich et al. (1993). Since this extrapolation was over a long range, small errors in determining the slope of the curves, known as the m values, can cause large differences in folding free energies. Under such circumstances, it is usually more accurate to assume that very closely related proteins have the same m value. Thus, we chose to use m values obtained by averaging those obtained from all the mutant proteins. However, the differences in slope between the oxidized and reduced forms of DsbA appear to be significant, possibly due to residual structure in the denatured form caused by the disulfide bond (Zapun et al., 1993). Therefore, we averaged the oxidized and reduced forms separately.

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