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The Influence of His94 and Pro149 in Modulating the Activity of *V. cholerae* DsbA

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ABSTRACT

DsbA is the primary catalyst of disulfide bond formation in the periplasm of gram-negative bacteria. Numerous theoretical and experimental studies have been undertaken to determine the molecular mechanisms by which DsbA acts as a potent oxidant, whereas the homologous cytoplasmic protein, thioredoxin, acts as a reductant. Many of these studies have focused on the nature of the two residues that lie between the active-site cysteines. Although these are clearly important, they are not solely responsible for the differences in activity between these thiol-disulfide oxidoreductases. Q97 in the helical domain of *E. coli* DsbA has been implicated in influencing the redox potential of *E. coli* DsbA. In *V. cholerae* DsbA, the analogous residue is H94. In this study, the effect of H94 on the oxidase activity of DsbA is examined, along with the role of the conserved cis-proline residue P149. The DsbA mutant H94L shows a nearly fourfold increase in activity over the wild-type enzyme. To our knowledge, this is the first time an increase in the normal activity of a thiol-disulfide oxidoreductase has been reported. Potential reasons for this increase in activity are discussed. *Antioxid. Redox Signal.* 5, 359–366.

INTRODUCTION

DsbA is one of a family of thiol-disulfide oxidoreductases present in the periplasm of gram-negative bacteria (for a recent review, see 7). DsbA is a potent oxidant and is thought to be the primary catalyst of disulfide bond formation in the periplasm. It was originally detected using genetic screens in which *dsbA*$^-$ mutants were found to exhibit defects in the formation of native disulfide bonds in newly synthesized periplasmic proteins (3, 34).

The structures of both *E. coli* and *V. cholerae* DsbA have been solved to high resolution and in different oxidative states (8, 14, 17, 22, 29). All of the structures reveal a (βα)βαβαββα thioredoxin fold (which is characteristic of many proteins involved in sulfur metabolism) into which is inserted an all α-helical domain. The -Cys-Xaa-Yaa-Cys- active-site residues, characteristic of the thiol-disulfide oxidoreductase superfamily, are located at the N-terminus of α2.

A significant number of studies have been performed experimentally and theoretically to explain why periplasmic DsbA is a potent oxidant, readily catalyzing the formation of disulfide bonds in peptides and proteins, whereas the homologous cytoplasmic protein, thioredoxin, is a potent reductant, readily catalyzing the reduction of disulfide bonds to free thiols (6, 11–13, 18, 19, 23, 24, 31, 32, 35). The primary focus of these studies has been on the residues that lie between the two active-site cysteines. For DsbA, there is a correlation between the reduction potential of mutant forms of the enzyme and the acidity of the N-terminal cysteine in the active-site motif; this in turn depends on the nature of the two residues between the active-site cysteines (as examples, see 12, 18). For thioredoxin, the correlation is not so simple (as example, see 23). A

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theoretical consideration of the application of the Nernst equation to the reduction potential of the active-site motif identified both pH-dependent and pH-independent components, but no single factor for the pH-independent component was identified (6). A molecular model for the redox potential difference between thioredoxin and DsbA has been developed based on electrostatic calculations from which it was predicted that the differences arise due to the additive effects of several side-chain and mainchain groups (11). Specifically H32 and Q97 of *E. coli* DsbA were singled out as having the greatest thiolate-stabilizing effect, whereas groups in the vicinity of the active site, E24, E37, K58, and P151, were also noted as potentially having an effect. An experimental investigation into the effects of E24, E37, and K58 led to the conclusion that the conserved charge network between these residues did not cause the high redox potential of the enzyme (15, 19), but see also the theoretical consideration of these results (30).

To date, much of the focus of research has been on redox potential, *i.e.,* the thermodynamics of the reaction, although some excellent and detailed kinetic studies for the wild-type enzyme have been undertaken (as example, see 9). These studies indicate that, *in vitro*, the rate-limiting step for DsbA-catalyzed peptide oxidation is reoxidation of the enzyme by oxidized glutathione (GSSG).

In this study, we investigated the role of H94 and P149 of *V. cholerae* DsbA (analogous to Q97 and P151 of *E. coli.* DsbA) on the kinetics of DsbA-catalyzed peptide oxidation. These residues are remote in sequence from the active-site disulfide, and the former is located within the helical B-domain. We demonstrate that mutations in H94 increase the rate of reoxidation of the enzyme and hence increase the rate of DsbA-catalyzed peptide oxidation up to fourfold. The physiological significance of this observation is discussed.

MATERIALS AND METHODS

Molecular biology: point mutations

The gene for wild-type *V. cholerae* had previously been cloned into the pTB361 (pUKC1072) and pTB375 (pUKC1076) expression vectors, which contain a tetracycline resistance marker (21). Mismatch PCR primers for the 5′-3′ direction were designed to introduce the mutations H94L, H94K, P149A, and P149G; the primers for H94 spanned a unique KpnI site, whereas those for P149 spanned a unique AgeI site. By using pUKC1072 DNA as template, each of these was combined in a PCR reaction with the 3′-5′ primer used previously for the amplification of the complete coding sequence. For the H94 mutagenesis, the PCR products (300 bp) were restricted with KpnI and BglII; for the P149 mutagenesis, the PCR products (100 bp) were restricted with AgeI and BglII. In both cases, the products were ligated with appropriately doubly-restricted pUKC1076 to generate the required mutant constructs for H94L (pUKC1077), H94K (pUKC1078), P149A (pUKC1079), and P149G (pUKC1080).

Molecular biology: B-domain-deletion construct

The protein sequence of full-length DsbA was aligned with those of thioredoxin and the thioredoxin-like **a** domain of protein disulfide isomerase (PDI). Compared with these, DsbA contains a major insertion (the B domain) that replaces the dipeptide sequence –SN- in thioredoxin and –EE- in PDI. In a model of the tertiary structure of *V. cholerae* DsbA, the B domain was deleted and replaced by a range of dipeptide and tripeptide linker sequences. Energy minimization suggested that linkers of the sequence –EXE- would be optimal. Of these, the linker –ELE- was favorable on energetic grounds and could also introduce a unique SacI restriction site. A fragment assembly approach was therefore used to construct a DsbA mutant in which the B domain was replaced with the sequence –ELE-. Primers were designed to be complementary with the regions of the DsbA A domain bordering the B domain junctions (*i.e.,* up to residue S59 and from residue D137 of the mature sequence, respectively) and also to encode the –ELE- insert including the SacI site. The 3′-5′ primer was combined in a PCR with the 5′-3′ primer used initially, and the product was doubly restricted with NdeI and SacI; the 5′-3′ primer was combined in a PCR reaction with the 3′-5′ primer used initially, and the product was doubly restricted with SacI and BglII. The purified restricted fragments were then combined in a triple ligation with NdeI/BglII-restricted pUKC1076 to generate the plasmid pUKC1075.

MBP-DsbA fusion protein production

dsbA was expressed as a fusion protein in *E. coli* using the *malE* fusion system, where the malE gene encodes maltose binding protein (MBP). For secretion of MBP-DsbA into the periplasm, the expression vector pMalp was used. Cytoplasmic expression was obtained by cloning into the expression vector pIH902 (New England Biolabs). By synthesizing specific primers, *dsbA* and *dsbA* Δ *B* were amplified by PCR in such a way that the amplified DNA fragment started with the GCT CAA TTT AAA . . . codons encoding the N-terminus of processed DsbA and ended a few base pairs after the TAA stop codon. This fragment was inserted at the single *Stu*I site of the pIH902 and pMalp plasmids, respectively. In the resulting *malE-dsbA* fusion, the *dsbA* GCT CAA TTT AAA . . . codons immediately followed the factor Xa cleavage sequence; hence, the cleaved DsbA constructs had authentic mature N-terminii. The correct orientation and sequence of *dsbA* and *dsbA* Δ *B* in the *malE* fusions were determined by DNA sequencing. All of the fusion proteins were expressed solubly.

Protein purification

Wild-type *V. cholerae* DsbA and mutants were prepared by the method of Lowe *et al.* (21). The concentration of DsbA was determined spectrophotometrically using a calculated absorption coefficient of 11,900 M^{-1} cm^{-1} at 278 nm.

MBP fusion proteins were purified using amylose affinity chromatography by the method of Riggs (27). DsbA Δ B was cleaved from MBP-DsbA Δ B using factor Xa and purified by anionic exchange chromatography on a HiTrap Q column (Pharmacia) and subsequent amylose affinity chromatography.

Spectroscopic determination of the oxidation of the substrate peptide

The method of Ruddock *et al.* (28) was used to determine the rate of oxidation of the substrate peptide NRCSQGSCWN. In essence, substrate peptide was added to a McIlvaine buffer

(0.2 *M* disodium hydrogen phosphate/0.1 *M* citric acid; pH 4.0–7.5) to which had been added appropriate amounts of reduced glutathione (GSH), GSSG, and enzyme and allowed to thermally equilibrate to 25°C for 3 min. The solution was mixed by inversion, and then the change in fluorescence intensity (excitation 280 nm, emission 350 nm, slits 5/5 nm) that accompanies oxidation of the peptide was monitored over an appropriate time (10 min to 1 h) and 600–900 data points collected.

EtxB biogenesis assay

E. coli heat-labile enterotoxin (Etx) is an AB_5 toxin. The intrachain disulfide bond (Cys^9-Cys^{86}) of EtxB is essential for the formation of the B-pentamers and absolutely requires DsbA in *V. cholerae* for its biogenesis and subsequent secretion into the culture medium (33). Plasmids expressing MBP-DsbA fusion proteins targeted to the periplasm were transformed into the *dsbA*⁻ strain *V. cholerae* UKC13 *dsbA::*Tn*phoA*.7A (pMMB107) [in *V.cholerae* UKC13, the chromosomal *ctx* genes encoding the cholera toxin are deleted; pMMB107 encodes EtxB] by electroporation. After induction with 1 m*M* isopropyl β-D-1-thiogalactoside (IPTG), the *malE-dsbA* fusions are expressed in *V. cholerae* and are secreted into the periplasm. Co(over)expression of *etxB* and *malE-dsbA* or other active MBP-DsbA fusion proteins leads to production and secretion of the B-pentamer.

Dithiothreitol (DTT) susceptibility assay

The *dsbA*⁻ *V. cholerae* strain shows an increased susceptibility to the external addition of the reductant DTT, compared with the wild-type strain, which can be relieved by the expression of active variants of DsbA. Differential growth of strains expressing MBP-DsbA fusion proteins on plates containing DTT is indirectly indicative of the thiol-disulfide oxidase activity of these fusion proteins. Each strain was grown to mid-log phase in LB media containing appropriate antibiotics, and then 2 μl of this was pipetted onto LB-agar plates containing the appropriate antibiotics and differing concentrations of DTT (from 3 to 10 m*M*). Growth was observed after overnight incubation at 37°C.

RESULTS

In vitro *analysis*

Protein purification of the wild-type and mutant forms of *V. cholerae* DsbA resulted in the generation of protein of the expected size, which was homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown).

Wild-type DsbA and the H94L, H94K, P149A, and P149G, DsbA mutants were active in catalyzing the oxidation of the 10-amino acid reporter peptide at pH 6.0 (data not shown). Subsequently, all five proteins were tested for the pH dependence of the catalysis of disulfide bond formation.

The rate of wild-type DsbA-catalyzed oxidation increased with increasing pH over the pH range 4.0–7.0 (Fig. 1A). As the observed rate comprises the sum of the enzyme-catalyzed rate and the noncatalyzed rate (which also increases with increasing pH), the pH dependence of the enzyme-catalyzed rate shows a maximum at pH 6.5.

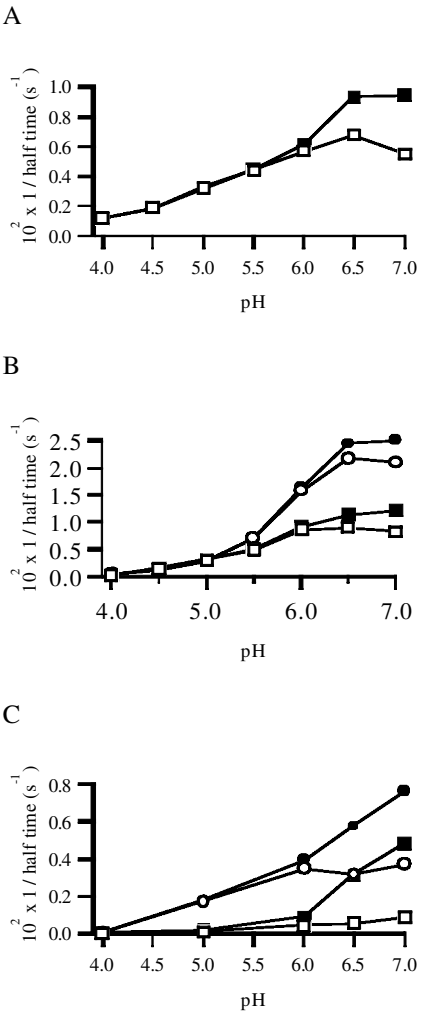

FIG. 1. pH dependence of the peptide-oxidase activity of wild type and mutant *V. cholerae* DsbA. Activities were determined in duplicate at each pH using 0.5 m*M* GSSG, 2 m*M* GSH, 300 n*M* enzyme, 5 μ*M* peptide substrate in McIlvaine buffer. (**A**) Wild-type enzyme, uncorrected (■) and corrected (□) for non–enzyme-catalyzed rate; (**B**) H94L, uncorrected (●) and corrected (○) for non–enzyme-catalyzed rate; H94K, uncorrected (■) and corrected (□) for non–enzyme-catalyzed rate. (**C**) P149A, uncorrected (●) and corrected (○) for non–enzyme-catalyzed rate; P149G, uncorrected (■) and corrected (□) for non–enzyme-catalyzed rate. Error bars are not shown for clarity; average error was 8% and error range 2–12%.

Likewise, the rate observed with the H94L and H94K DsbA mutants also increased with increasing pH over the pH range 4.0–7.0 (Fig. 1B). Correcting for the noncatalyzed rate, the pH dependence of the H94L mutant shows a maximal rate at pH 6.5, whereas the pH dependence of the reaction catalyzed by the H94K mutant shows the formation of a more distinct plateau region (with the activity at pH 6.0 only slightly lower than that at pH 6.5). Both of the mutations in H94 result in proteins that are more active than wild type at pH values of 5.5–7.0, but less active at pH values of 4–5 (see Fig. 2). The activity of H94K was 132% of wild type at pH 6.5 (wild-type optimal activity) and maximally 151% of wild type (at pH 7.0). The corresponding figures for the H94L mutant were 324% and 383%, respectively.

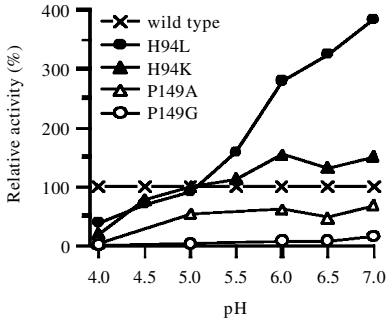

FIG. 2. Relative activities of the H94L, H94K, P149A, and P149G mutants of DsbA to the wild-type enzyme as a function of pH. Activities were determined in duplicate at each pH using 0.5 m*M* GSSG, 2 m*M* GSH, 300 n*M* enzyme 5 μ*M* peptide substrate in McIlvaine buffer.

Both the P149A and P149G mutants showed only marginal activity at pH 4.0. Over the pH range 5.0–7.0, both mutants showed an increasing rate of catalysis with increasing pH (see Fig. 1C). The P149A DsbA mutant appeared to show a plateau in activity over the pH range 6–7, whereas the P149G mutant increased in activity over the whole pH range tested. The activity of P149A was 47% of wild type at pH 6.5 and maximally 67% of wild type (at pH 7.0). The corresponding figures for the P149G mutant were 8% and 16%, respectively (see Fig. 2). These results are consistent with those for the analogous P151A mutant in *E. coli* DsbA, which was shown to have a trans peptide bond at this position in the mutant (cis in wild type) and to display less than half of the activity of wild type using alkaline phosphatase biogenesis as an *in vivo* reporter assay (5, 25).

In vivo *analysis*

V. cholerae dsbA⁻ strains expressing differing MBP-DsbA fusion proteins were also tested for their ability to grow on plates containing differing concentrations of the reductant DTT. pMalp and a vector expressing the enzymatically inactive C30A variant of DsbA acted as negative controls. The results (see Table 1) indicate that both H94 mutants were able to replace wild-type DsbA *in vivo*, whereas the strain expressing the P149A mutant showed greatly increased susceptibility to DTT (close to the negative control), indicating decreased *in vivo* activity. The DTT sensitivity assay that is widely used to screen for *in vivo* activity is not a direct measure of DsbA activity, but more likely a measure of disulfide bond formation in membrane proteins, for which DsbA is required. There is no published correlation between the level of DsbA expression and DTT sensitivity, and even constitutive/low levels of DsbA probably give maximal protection. Hence, the results obtained from this assay are consistent with the *in vitro* data.

TABLE 1. GROWTH OF *DSBA*⁻ *V. CHOLERAE* STRAINS EXPRESSING MBP-DSBA FUSIONS ON PLATES CONTAINING INCREASING CONCENTRATIONS OF DTT

Construct	*Good growth (m*M)	*Poor growth (m*M)
pMalp	4	7
MBP-DsbA C30A	4	7
MBP-DsbA Δ B	4	7
MBP-DsbA P149A	5	8
MBP-DsbA	8	10
MBP-DsbA H94L	8	10
MBP-DsbA H94K	8	10

The other type of *in vivo* assay commonly used is the ability of expressing strains to synthesize a coexpressed protein that contains disulfide bonds essential for its folding and/or activity. Accordingly, we tested the strains expressing differing MBP-DsbA fusion proteins for their ability to make the B-subunit of heat-labile Etx, a protein that absolutely requires DsbA activity in its biogenesis (33). Both H94 mutants and the P149A mutant produced levels of EtxB equivalent to wild type, whereas no EtxB production was seen for the pMalp or C30A negative controls, all MBP-DsbA fusions being expressed to comparable levels (data not shown). As the MBP-DsbA fusion system produces very large amounts of protein when induced, the sensitivity of the EtxB production tested to small changes in DsbA activity is poor, resulting in no differences being observed with the P149A mutant in this assay despite reduced activity in other assays.

Generation of a single-domain DsbA

DsbA is a two-domain protein, one domain being thioredoxin-like and containing the active site, the other, B domain, is an all α-helical insert that is found in DsbA uniquely in the superfamily. As H94 is located in the B domain and is the only residue to date in that domain that has been implicated in modulating the activity of DsbA, it was decided to test the *in vivo* and *in vitro* activities of a DsbA Δ B mutant, *i.e.,* a mutant in which the B domain had been entirely replaced by a tripeptide linker.

DsbA Δ B was produced as a MBP fusion protein and purified to homogeneity by amylose affinity chromatography (see Fig. 3). DsbA Δ B was generated from this fusion protein by

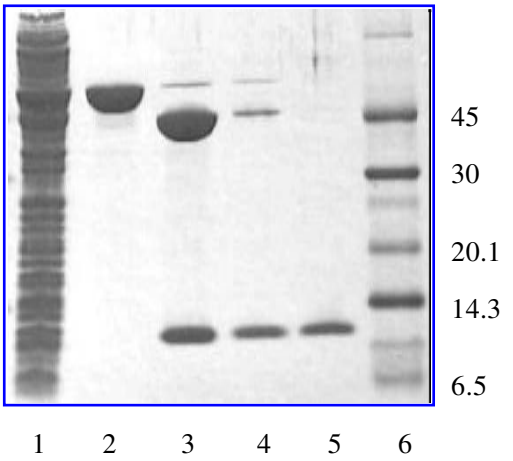

FIG. 3. Purification of MBP-DsbA Δ B and DsbA Δ B. The MBP-DsbA Δ B fusion protein was purified from crude lysate (lane 1) by amylose affinity chromatography. The purified fusion protein MBP-DsbA Δ B (lane 2) was cleaved by factor Xa (lane 3). MBP-DsbA Δ B, MBP, and DsbA Δ B were separated by anionic exchange chromatography on a HiTrap Q column. The DsbA Δ B-containing fractions (lane 4) were then applied to a second amylose affinity chromatography to remove traces of MBP. Purified DsbA Δ B was found in the wash through of the column (lane 5). Lane 6, molecular weight standards.

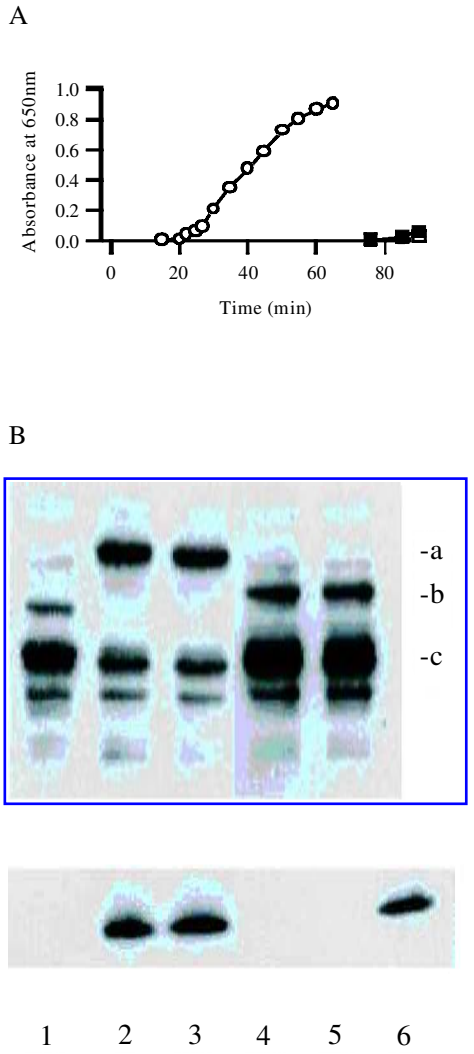

FIG. 4. Determination of the activity of MBP-DsbA and MBP-DsbA Δ B. (**A**) *In vitro* activity. The activities of MBP-DsbA (○), MBP-DsbA C30A (●, negative control), and MBP-DsbA Δ B (□) were compared using the insulin reduction assay (buffer, ■) according to the methodology of Holmgren (16). Identical amounts of the fusion proteins MBP-DsbA and MBP DsbA C30A were used in the assay. MBP-DsbA Δ B was used at approximately double the concentration. (**B**) *In vivo* activity. The plasmids pMalp (lane 1, control), pMalp *dsbA* (lanes 2 and 3), and pMalp *dsbA* Δ *B* (lanes 4 and 5) were transformed into *V. cholerae* UKC13 *dsbA::*Tn*phoA*.7A (pMMB107). The expression both of *etxB* and *malE-dsbA* (*malE-dsbA* Δ *B*) was induced by the addition of 1 m*M* IPTG to the *V. cholerae* cells at an optical density of A_{600} = 0.25. Two and a half hours after induction, comparable aliquots of the cell extracts and the culture supernatants, respectively, were subjected to SDS-PAGE and immunoblotting. The culture supernatants were applied in Laemmli buffer to the gels, but without prior heating. MBP-DsbA fusion proteins in the cell extracts were detected using a polyclonal anti-MBP antibody [upper panel; the molecular weights of MBP-DsbA fusion protein (a), MBP-DsbA Δ B fusion protein (b), and MBP (c) are indicated]. The 55-kDa EtxB pentamer was detected using monoclonal anti-$EtxB_5$ antibodies (lower panel; lane 6 is purified EtxB control).

factor Xa cleavage and purified by anionic exchange chromatography and amylose affinity chromatography (see Fig. 3). The N-terminus of the purified DsbA ΔB was verified by Edman degradation and its mass verified by electrospray mass spectrometry (12153.0; compared to the expected mass of 12155.7 for the reduced protein, the difference in mass being within the experimental error of the technique used). No activity was observed in the fluorescence peptide-oxidase assay for either the MBP-DsbA Δ B fusion or for DsbA Δ B (data not shown), nor was activity observed for the MBP-DsbA Δ B fusion in the insulin reduction assay (see Fig. 4A). Furthermore, *in vivo* the strain expressing the DsbA Δ B fusion protein showed identical DTT sensitivity as the pMalp and DsbA C30A negative controls, and no EtxB production was observed (see Fig. 4B).

DISCUSSION

Previous studies using a 10-amino acid synthetic peptide demonstrated that the pH dependence of catalysis of peptide oxidation by wild-type *V. cholerae* DsbA showed a maximum at pH 6.5 (28), a result confirmed here. The pK_{app} values for a "two-ionizations" model of these data gave values of 5.1 and 7.5. The total change in fluorescence, *i.e.,* the degree of peptide oxidation, was observed to be pH-independent, confirming that only the kinetics of the reaction are being modulated by pH. The rate-limiting step for the reaction is reoxidation of reduced DsbA by GSSG; more specifically, it is the nucleophilic attack by the N-terminal active-site Cys residue (as a thiolate) on GSSG, to form the enzyme-glutathione mixed disulfide.

As the thiolate form of a cysteine side-chain would be expected to be more reactive in this reoxidation step than the free thiol, it was suggested that the pK_{app} value of 5.1 could represent the pK_a of the N-terminal active-site cysteine of *V. cholerae* DsbA (28). Alternatively, this, and/or the higher pK_{app}, may reflect the pK_a of a group close to the active site that influences reactivity. Given that the pK_a values for the active-site cysteines of DsbA have been reported to be 3.5 and >10.0 (26), we sought to identify other residues that might be responsible for the observed ionizations at pH 5.1 and 7.5, and to test for their impact on the reaction kinetics and pH dependence.

Previous theoretical studies on DsbA have indicated that many side-chain and main-chain groups acting synergistically may influence the ionization equilibria of the active-site cysteines and hence the redox potential of DsbA. Three potential groups with pK_a values that may lie in this range are E24, H32, and H94. Mutation of E24 in *E. coli* DsbA to glutamine resulted only in a minimal change in the redox potential, structural stability, and pK_a of the active-site cysteine (19), suggesting that E24 in the wild-type protein is in the uncharged acid form in neutral conditions. Consistent with this speculation, D26 of thioredoxin, the homologous residue to E24, has been reported to have a pK_a of 7.5 (20). We deduced that E24 of DsbA was likely to be responsible for the higher pK_{app} transition observed at pH 7.5 in our studies, and we focused attention on the lower transition at pH 5.1.

To investigate whether the pH dependence of activity in the acid region up to pH 6.5 was dependent on H94, this residue

was mutated to lysine and to leucine. In the case of both mutations, an increase in oxidase activity compared with wild type was observed at pH values greater than 5.5. For both mutants, the change in fluorescence upon oxidation of the peptide over the reaction time course was linear for >50% of the reaction, implying that the rate-limiting step for the reaction is the reoxidation of DsbA, as has previously been described for wild-type DsbA (9, 28). If the ionization of the side chain of H94 were directly responsible for the pH dependence of reaction rate in the acid range, then conversion of this side chain to uncharged Leu or positively charged Lys would be expected to abolish the pH-dependent variation in kinetics in this pH range. Instead we observed that the pH dependence of the activity was retained in both mutants as for the wild type; however, the pH at which half-maximal activity is observed was 5.1 for wild type, 5.4 for the H94K mutant, and 5.7 for the H94L DsbA mutant. The retention of this pH dependence in both of the H94 mutants makes it clear that neither pK_{app} for the wild-type enzyme directly represents the pK_a of this residue. These results are therefore consistent with the hypothesis that the pK_{app} of 5.1 for the wild-type *V. cholerae* enzyme represents the N-terminal active-site cysteine (28), although this is significantly higher than the reported pK_a of 3.5 determined by UV spectroscopy for *E. coli* DsbA (26). Nevertheless, our data indicate that the protonation state of H94 influences the pK_a of the relevant group This confirms the suggestion (11) that the juxtaposition of H94 can lower the pK_a of the active-site cysteine, and further implies that the positively charged lysine in the H94K mutant is at nonoptimal geometry to fulfill the same role.

Furthermore, these results are consistent with the idea that residues adjacent to the active site can influence the pK_a of the active-site cysteine, and hence the pK_a of the active site cysteine will vary as a function of pH as adjacent groups are protonated/deprotonated and as pH-dependent conformational changes occur [*V. cholerae* DsbA undergoes a significant conformational change below pH 4.0 that completely inactivates it (Ruddock and Freedman, unpublished results)]. The pK_a values of the active-site cysteines may also vary with changes in conformation and/or protonation state during the catalytic cycle. From these results, it is apparent that H94 does play a small role in altering the pK_a of the active-site cysteine; this leads to an increased redox potential through the linkage of the ionization and redox equilibria, but it also decreases the kinetics of reoxidation *in vitro* compared with alternative residues.

In vivo, DsbA is not reoxidized by GSSG (which is not present in the periplasm), but by the inner membrane protein DsbB, which derives its oxidizing potential from the electron transport system via quinones (1, 2, 4). However, we believe that the observations we report here do have a physiological significance. A strongly oxidizing active-site disulfide (in terms of equilibria and standard redox potential) will destabilize the protein, *i.e.,* the dithiol state of the enzyme needs to be more stable than the disulfide (32). The linkage of ionization and redox equilibria has the consequence that features that stabilize the N-terminal cysteine as a thiolate favor the dithiol form of the active site over the disulfide form, and hence generate a more strongly oxidizing enzyme. This occurs in DsbA, and hence the N-terminal active-site cysteine in DsbA has been reported to have a pK_a of ~3.5 (26), significantly lower than that of the equivalent cysteine in thioredoxin, which is reported to be ~7.1 (10). Our suggested value of 5.1 for this p*K* is significantly lower than for the equivalent residue in thioredoxin and much lower still than the values observed in model thiols. However, a surface exposed thiolate in the complex environment of the periplasm will be prone to numerous potential chemical reactions, including the ability of the thiolate to act as a nucleophile against disulfide bonds in proteins that have yet to attain their native structure. This would be counterproductive to the physiological function of DsbA. This would potentially be much more of a problem for the periplasmic thiol-disulfide oxidoreductase DsbA than the endoplasmic reticulum resident members of the family, such as PDI, as the periplasm is not a highly regulated milieu due to the porosity of the outer membrane, which allows free diffusion of species smaller than 600 Da in size. It is possible that the all helical B domain, which uniquely among the superfamily is inserted into the thioredoxin-like domain, evolved not in response to the need to modulate the redox potential of the enzyme, but rather to protect the highly reactive active-site that is generated in an enzyme with a strong oxidizing potential. Conversely, one effect of this "protection" would be to diminish the rate of reaction of DsbA in its oxidative activity.

To examine this possibility, the *in vitro* and *in vivo* activities of DsbA Δ B, *i.e.,* a mutant lacking the B domain, were examined. Although near equivalent amounts of the MBP-DsbA Δ B fusion mutant to MBP-DsbA could be detected, no protection from the effects of the reductant DTT were observed nor were detectable amounts of EtxB generated, indicating that the DsbA Δ B construct was inactive *in vivo* as an oxidase. This was confirmed *in vitro* using the fluorescence peptide oxidase assay, and its lack of thiol-disulfide oxidoreductase activity was also observed in the insulin reduction assay. Although modulation of the linker region between the two halves of the thioredoxin-like domain may potentially have an effect on the structure of DsbA Δ B and hence on the observed activity, our initial results suggest that our DsbA-Δ B construct is folded and that the B domain is essential for the activity of DsbA. If the B domain originally evolved to protect the highly reactive active-site thiolate, it now clearly has additional functions as well.

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ABBREVIATIONS

DTT, dithiothreitol; EtxB, *E. coli* heat-labile enterotoxin B subunit; GSH, reduced glutathione; GSSG, oxidized glutathione; IPTG, isopropyl β-D-1-thiogalactoside; MBP, mal-

tose-binding protein; PDI, protein disulfide isomerase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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