DbSC, a member of the Dsb family in the periplasm of Gram-negative bacteria, is not only a disulfide isomerase but also a chaperone. Five DsbC mutants with Cys in the active site sequence of Cys-Gly-Tyr-Cys and the nonactive site disulfide Cys-Cys replaced by Ser have been studied. The results show that the active site Cys residues are necessary for enzyme activity but not required for chaperone activity, while the lack of the nonactive site disulfide results in a decreased chaperone activity in assisting the reactivation of denatured α-glyceraldehyde-3-phosphate dehydrogenase but has no effect on enzyme activities. Wild-type DsbC was overexpressed and correctly processed as a soluble periplasmic protein. Mutation in one of these Cys residues results in aggregation or extracellular/membrane localization, but does not affect the proper processing. DsbC mutated in either Cys residue of nonactive site disulfide shows higher sensitivity to unfolding by guanidine hydrochloride and slower refolding compared with wild-type DsbC and the active site Cys mutants. The above results provide experimental evidence for structural role of the nonactive site disulfide in folding and biological activities of DsbC.

DbC family proteins, comprising at least six members (DsbA, DsbB, DsbC, DsbD, DsbE, and DsbG), have been characterized to be thiol oxidoreductases in the periplasm of prokaryotic cells and responsible for the formation of disulfide bonds in newly synthesized proteins (1–3). Among the Dsb family, DsbC, a homodimer, is homologous, to the highest extent, with eukaryotic protein-disulfide isomerase (PDI) in terms of biological properties and functions (4–8). PDI has been characterized to be not only an isomerase but also a chaperone (9, 10). Recently we have reported that DsbC shows even more pronounced chaperone activity than PDI in promoting the in vitro reactivation and suppressing aggregation of denatured α-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) during refolding (11), whereas DsbA shows a weaker chaperone activity than that of PDI (12). DsbG, strongly homologous to DsbC, has also been characterized to have chaperone activity (13). The crystal structure of DsbC reveals that it is a V-shaped molecule with each arm of the V, a monomer consisting of a C-terminal thioredoxin-domain and a N-terminal association domain connected by a hinged linker (14). DsbC has four cysteines in each 23-kDa subunit. Two in the active site (Cys-Gly-Tyr-Cys) and Cys protruded out of the molecule are responsible for the oxidoreductase activity (6). Single replacements of Cys with different residues all result in complete loss of enzyme activities; however, the contributions of Cys to the biological activities were reported diversely (5, 15). The nonactive site cysteines, Cys and Cys, were reported to form a stable disulfide bond somehow buried and disrupted only in the presence of large excess of dithiothreitol (DTT) (6, 16). Among the members of the thioredoxin superfamily characterized so far, only DsbC has a nonactive site disulfide bond, Cys-Cys, which was suggested to play a purely structural role (6, 17). Recent crystal structure analysis shows that this unique disulfide bond located in a helical insert within the C-terminal catalytic domain is partially solvent exposed (14).

DsbC, like other oxidoreductases, exists in both oxidized and reduced forms. Reduced DsbC is the functional and dominating form (8, 15, 16) and is regenerated from oxidized form by membrane-bound DsbD (8, 15, 18). Overexpression of DsbC results in the accumulation of oxidized form due to exceeding the reductive capacity of DsbD (15). Unlike DsbC, reduced and oxidized DsbC show the same intrinsic fluorescence and CD spectra (6).

In this paper we report that five DsbC mutants, with Cys replaced by Ser, overproduced in Escherichia coli using a Histag signal to direct export as described in our previous work (21), show different properties in terms of folding, export, solubility, stability, and biological activities compared with wild-type DsbC.

**EXPERIMENTAL PROCEDURES**

**DNA Construct**—Primers designed for mutations of Cys at the positions of 98, 101, 141, and 163 of DsbC into Ser are listed in Table I. The plasmid BS-DsbC was constructed by insertion of the coding sequence of DsbC precursor amplified from the plasmid pDsbC, a generous gift from Dr. Rudi Glockshuber (Eidnössische Technische Hochschule, Hönegg, Switzerland), into Bluescript II/SK (+) (Stratagene) as described previously (21). The preparation of single-stranded DNA of BS-DsbC and oligonucleotide-directed mutagenesis in vitro were performed essentially according to Carter (22). The full-length coding sequences of the corresponding precursors of the five mutants were confirmed with both strands, and then subcloned into pQE-30 vector (Qiagen) via BamHI site to create corresponding expression plasmids pQE-C98S, pQE-C98S/C101S, pQE-C101S/C163S, pQE-C141S, and pQE-C163S according to Liu et al. (21), resulting in modified precursors with a MRGSH GS-fused signal sequence at the N termini (Table I).

**Expression and Purification**—Transformed M15 [REP4] cells (Qiagen) were grown in 2× YT media with 100 μg/mL ampicillin and 25 μg/mL kanamycin. The overnight culture was diluted 100-fold and incubated at 37 °C for 2 h followed by induction with isopropyl β-D-thiogalactopyranoside (IPTG) of different concentrations from 0.001 mM to 1 mM. Osmotic shock was used to release soluble periplasmic proteins, and the shocked cells were sonicated and centrifuged at 35000 rpm for 90 min at 4 °C. The pellets were treated with 1% Triton X-100 in 50 mM Tris buffer (pH 8.0) to release membrane-bound proteins.

This paper is available online at http://www.jbc.org/
Expression and Processing of Wild-type and Mutant DsbC Proteins—As shown in Fig. 1, precursors and processed species of wild-type DsbC and mutants move with significantly different mobility in SDS-PAGE (12%). Induced with IPTG at low concentrations of 0.001 and 0.01 mM, all proteins were efficiently processed, and higher concentrations of IPTG (≥0.1 mM) resulted in the accumulation of unprocessed species. Wild-type DsbC was overexpressed in the periplasm as a soluble protein, consisting 20–30% of the total cellular proteins. C98S/C101S, similar to wild-type DsbC, appeared in the periplasm as a soluble protein when induced for 4 h, but all secreted or leaked into the medium if induced overnight (data not shown). More than 180 mg of processed C98S/C101S was obtained from 1 liter of cell-free culture. Processed species of C98S was partially released by osmotic shock while those of C141S, C163S, and C101S/C163S totally aggregated. Decreasing the yield of solubilized proteins before (B) and after induction with IPTG of 1.0, 0.1, 0.01 and 0.001 mM, respectively (lanes 1–4); proteins in supernatant (lanes 5–8) and in osmotically shocked cells (lanes 9–12) after osmotic shock corresponding to lanes 1–4, respectively. Arrows indicate the positions of precursors.

Expression and Processing of Wild-type and Mutant DsbC Proteins—As shown in Fig. 1, precursors and processed species of wild-type DsbC and mutants move with significantly different mobility in SDS-PAGE (12%). Induced with IPTG at low concentrations of 0.001 and 0.01 mM, all proteins were efficiently processed, and higher concentrations of IPTG (≥0.1 mM) resulted in the accumulation of unprocessed species. Wild-type DsbC was overexpressed in the periplasm as a soluble protein, consisting 20–30% of the total cellular proteins. C98S/C101S, similar to wild-type DsbC, appeared in the periplasm as a soluble protein when induced for 4 h, but all secreted or leaked into the medium if induced overnight (data not shown). More than 180 mg of processed C98S/C101S was obtained from 1 liter of cell-free culture. Processed species of C98S was partially released by osmotic shock while those of C141S, C163S, and C101S/C163S totally aggregated. Decreasing the yield of synthesis by using 0.001 mM IPTG did not improve the solubility. All five mutants show the same N-terminal sequence of Asp-Asp-Ala as that of wild-type DsbC, confirming that the signal sequences in all mutants have been processed correctly.

Subcellular Location of Processed Mutants—As shown in Table II, the supernatant (S1) after osmotic shock showed almost all cellular β-lactamase activity, indicating that the proteins in the supernatant fraction prepared by the method of osmotic shock are indeed located in the periplasm. The activi-

### Table I

| Primer | Sequence |
|-------|----------|
| P1    | 5'-ttttactgttacttggtgccctaga-3' |
| P2    | 5'-ttttactgttacttggtgccctaga-3' |
| P3    | 5'-atggtattctccttccctagac-3' |
| P4    | 5'-gtcgcagctccagatgagtccgc-3' |

Proteins obtained at each step were analyzed by SDS-PAGE (12%). β-Lactamase (23, 24), NADH oxidase (24), and glucose 6-phosphate dehydrogenase (25) were used as markers to assign subcellular-compartments of periplasm, inner membrane, and cytoplasm of M15[REP4] cells transformed with plasmid pQE-30, respectively.

### RESULTS

#### Expression and Processing of Wild-type and Mutant DsbC Proteins—As shown in Fig. 1, precursors and processed species of wild-type DsbC and mutants move with significantly different mobility in SDS-PAGE (12%). Induced with IPTG at low concentrations of 0.001 and 0.01 mM, all proteins were efficiently processed, and higher concentrations of IPTG (≥0.1 mM) resulted in the accumulation of unprocessed species. Wild-type DsbC was overexpressed in the periplasm as a soluble protein, consisting 20–30% of the total cellular proteins. C98S/C101S, similar to wild-type DsbC, appeared in the periplasm as a soluble protein when induced for 4 h, but all secreted or leaked into the medium if induced overnight (data not shown). More than 180 mg of processed C98S/C101S was obtained from 1 liter of cell-free culture. Processed species of C98S was partially released by osmotic shock while those of C141S, C163S, and C101S/C163S totally aggregated. Decreasing the yield of synthesis by using 0.001 mM IPTG did not improve the solubility. All five mutants show the same N-terminal sequence of Asp-Asp-Ala as that of wild-type DsbC, confirming that the signal sequences in all mutants have been processed correctly.

#### Subcellular Location of Processed Mutants—As shown in Table II, the supernatant (S1) after osmotic shock showed almost all cellular β-lactamase activity, indicating that the proteins in the supernatant fraction prepared by the method of osmotic shock are indeed located in the periplasm. The activi-
ties of glucose-6-phosphate dehydrogenase and NADH oxidase mainly appeared in the fractions of sonicated supernatant (S2) and Triton X-100 extract (S3), respectively, indicating that S2 and S3 were mainly from cytoplasm and membranes, respectively. Therefore, as shown in Fig. 2 with the optimal induction of 0.01 mM IPTG for 6 h at 37 °C, C98S was expressed partly as a periplasmic soluble protein, partly as a membrane-bound protein, and partly as inclusion bodies in the periplasm. Although C163S and C101S/C163S totally formed inclusion bodies in the periplasm as they appeared only in the pellet of each preparation step, C141S mainly formed inclusion bodies in the periplasm with a small part as a membrane-bound protein.

Proteinase K Accessibility of the Osmotically Shocked Cells—As shown in Fig. 3, after digestion with proteinase K, the bands of the mutants C98S, C101S/C163S, C141S, and C163S in the osmotically shocked cells all disappeared and no new bands with lower molecular weight appeared even detected with Western blot, providing further evidence that the insoluble mutants were indeed located in the periplasm and the membrane-bound C98S and C141S were not imbedded inside the membranes but likely peripheral inner membrane proteins toward the periplasm. Many proteins sequestered in aggregates become highly resistant to proteolysis, but this is not the case for C98S, C141S, C163S, and C101S/C163S, suggesting that they are in a protease-accessible conformation.

Effect of Reductants on the Formation of Cys141-Cys163 Disulfide—The presence of 5 mM DTT or 10 mM N-acetylcysteine in the culture does not affect the processing, export, solubility, and activity of DsbC (Fig. 4A and Table III). Under nondenaturing condition, S-carboxymethylated products of DsbC from the strains grown in the media with DTT or N-acetylcysteine showed three bands with net negative charge of 0, 1, and 2, corresponding to the oxidized DsbC, heterodimer of oxidized and reduced subunits, and reduced DsbC, respectively (Fig. 4B), and showed about 30% of reductase activity of DsbC (Table III). The activity might be contributed by the Cys141 originally in the oxidized form and not alkylated in both oxidized DsbC and oxidized subunit of the heterodimer. The S-carboxymethylated products prepared in the presence of 6 M GdnHCl showed three bands with net negative charge of 0, 2, and 4, respectively, because of the alkylation of Cys101, which was not attacked by iodoacetic acid under nondenaturing condition. The above indicates that the nonactive site cysteines, Cys141 and Cys163, form a disulfide bond even with the cells grown in a reducing culture.

As shown in Fig. 5, under nondenaturing condition, the Cys141-Cys163 disulfide bond of C98S/C101S was only reduced by a large excess of the strong reductant DTT but not by reduced glutathione (GSH), indicating that this disulfide, although partially solvent-exposed (14), is stable in a reductive environment.

Enzyme Activities—As shown in Table III, compared with wild-type DsbC, C98S and C98S/C101S are devoid of enzyme activities and C101S/C163S shows approximate 30% reductase activity and 60% activity for catalyzing the oxidative refolding of RNase A. C141S and C163S show full enzyme activities of DsbC.

Chaperone Activity—As shown in Fig. 6, C98S and C98S/C101S, although inactive as enzymes, show the same ability as that of wild-type DsbC to assist the in vitro reactivation of denatured GAPDH to 30% at molar ratios larger than 15. C141S and C163S at high concentrations stimulate the reactivation of GAPDH to a lower extent of 15%, and C101S/C163S to only 10%.

Unfolding and Refolding—C98S, C98S/C101S, C141S, C163S, and C101S/C163S all show similar CD spectra, the same intrinsic fluorescence maximal emission wavelength of 312 nm and the same retention time on a Superose 12 HR 10/30 column as that of wild-type DsbC (data not shown), indicating that the mutations do not affect the secondary structure in general and the dimerization of the subunits.

As shown in Fig. 7, the Cm values, GdnHCl concentrations at the midpoints of unfolding curves measured by the ellipticity at 222 nm, of DsbC, C98S, and C98S/C101S are 2.19, 2.16, and 1.95 M, respectively, which are apparently higher than 1.46, 1.47, and 1.08 M for C141S, C163S, and C101S/C163S, respec-
DsbC were diluted 100-fold in the absence (N) or presence of 5 mM DTT (D) or 10 mM N-acetylcysteine (C), followed by incubation for 2 h, and then induced with 0.01 mM IPTG at 37 °C for 6 h. Panel A (12% SDS-PAGE), DsbC (lanes 1–3) from the cells cultured in different media as indicated was a combination of two peaks from the Q-Sepharose Fast Flow; total cellular proteins before (lane 4) and after induction (lanes 5–7); supernatants after osmotic shock (lanes 8–10); and osmotically shocked cells (lanes 11–13). Panel B (6% native-PAGE), DsbC from the cells cultured in the presence of different reductant as indicated was S-carboxymethylated in 0.1M Tris-HCl (pH 8.8) containing 5 mM EDTA, 1.8 mM GSSG, and 0.3 mM DTT, 0.05 mg/ml reduced and carboxymethylated DsbCN, DsbCD, and DsbCC with net charge of 0, 1, and 2 under non-denaturing condition, respectively, suggesting that C98S and C98S/C101S appear to be as stable to GdnHCl denaturation as DsbC, while the proteins with the nonactive site Cys residues mutated are less stable. The above suggest that the nonactive site disulfide Cys141-Cys163 indeed plays an important structural role in the stability of the DsbC molecule. Moreover, the $C_m$ values appear to be parallel to the solubility of the mutants in the periplasm; the higher the $C_m$, the more stable and soluble the proteins.

As shown in Fig. 8A, the refolding rates of denatured C98S and C98S/C101S upon dilution as monitored by the fluorescence emission intensity at 312 nm are the same as that of wild-type DsbC, and much faster than that of C101S/C163S, C141S, and C163S, suggesting that the formation of the nonactive site disulfide bond may accelerate the folding of DsbC.

Table III

| Protein | Insulin reduction $^b$ | Oxidative refolding $^c$ $(k_{cat})$ |
|---------|----------------------|-----------------------------------|
|         | $\Delta OD_{405}/min^2 \times 10^{-3}$ | min$^{-1}$ |
| Oxidized DsbC | 3.97 | 0.042 |
| DsbCN | 4.06 | 0.039 |
| DsbCD | 4.27 | 0.038 |
| DsbCC | 4.23 | 0.037 |
| m-DsbCN | 1.41 | 0.002 |
| m-DsbCD | 1.44 | 0.002 |
| m-DsbCC | 1.45 | 0.002 |
| C98S | 0 | 0 |
| C98S/C101 | 0 | 0 |
| C98S/C101S/C163 | 1.30 | 0.024 |
| C141S | 4.26 | 0.036 |
| C163S | 4.39 | 0.037 |

$^a$ DsbCN, DsbCD, and DsbCC were prepared as described in the legend to Fig. 4A for N, D, and C of lanes 1, 2 and 3 respectively; m-DsbCN, m-DsbCD, and m-DsbCC as described in the legend to Fig. 4B for S-carboxymethylated DsbCN, DsbCD, and DsbCC with net charge of 0, 1, and 2 under non-denaturing condition, respectively.

$^b$ Reduced activity of 2 mM protein determined in 0.1M potassium phosphate buffer (pH 7.0), containing 2 mM EDTA, 0.35 mM DTT, and 135 mM insulin.

$^c$ Oxidative activity determined in 0.1M Tris buffer (pH 7.5), with 2 mM EDTA, 1.8 mM GSSG, and 0.3 mM DTT, 0.05 mg/ml reduced and denatured RNase A. The data were expressed as averages of two independent experiments.

Fig. 7. Unfolding of wild-type and mutant DsbC proteins by GdnHCl. GdnHCl-induced transitions of oxidized DsbC (●), C98S (○), C98S/C101S (×), C141S (■), C163S (□) and C101S/C163S (△) at different molar ratios to GAPDH. The reactivation mixture was first kept on ice for 30 min and then at 25 °C for an additional 3 h before an aliquot containing 2 μg of GAPDH was taken for activity assay at 25 °C. The data were the averages of two independent experiments.

Fig. 8A. The refolding rates of denatured C98S and C98S/C101S upon dilution as monitored by the fluorescence emission intensity at 312 nm are the same as that of wild-type DsbC, and much faster than that of C101S/C163S, C141S, and C163S, suggesting that the formation of the nonactive site disulfide bond may accelerate the folding of DsbC.

Fig. 8B. Denatured and reduced DsbC, C98S, and C98S/C101S refolded markedly slower than the corresponding non-reduced species (Fig. 8B), providing further support to the above suggestion.
The DsbC subunit consists of a C-terminal thioredoxin-like domain with a CGYC-motif as the active site and a unique nonactive site disulfide in a helical subdomain and N-terminal association domain for dimerization (14). The present results have indicated that, as in eukaryotic PDI (30), the -CXYC-motif of DsbC is necessary for enzyme activities but not required for chaperone activity; thus, the chaperone activity of DsbC is independent of the active site. Mutants C98S and C98S/C101S with Cys\(^{101}\) mutated lose enzyme activities completely but retain full chaperone activity, while C101S/C163S with Cys\(^{101}\) mutated retains partial enzyme activities, indicating that Cys\(^{98}\) is essential whereas Cys\(^{101}\) is relatively less important for DsbC to function as a thiol oxidoreductase. It was reported that a single mutation of Cys\(^{101}\) to Ser resulted in decrease of \(\textit{in vivo}\) biological activities (16); however, a C101V mutant showed no activity in catalyzing the reduction of insulin (5). Cys\(^{98}\) has been suggested to facilitate rapid disruption of mixed disulfides with substrate, allowing efficient scanning of several disulfide isomers and preventing trapping of DsbC in disulfide-linked complexes as in the case of PDI (31).

The nonactive site cysteines form a stable disulfide bond, Cys\(^{141}\)-Cys\(^{163}\), which is resistant to the physiological reductant GSH at higher than \(\textit{in vivo}\) concentrations, and the formation of the disulfide bond is not affected by the presence of strong reductant DTT in culture. This disulfide is not required for enzymatic activity as C141S and C163S are fully active as a thiol oxidoreductase; however, the lack of this disulfide bond results in markedly decreased chaperone activity. Compared with both the wild-type DsbC and the mutants with intact Cys\(^{141}\)-Cys\(^{163}\) disulfide, the mutants C141S, C163S, and C101S/C163S, with no Cys\(^{141}\)-Cys\(^{163}\) disulfide, show higher sensitivity to GdnHCl-denaturation with lower \(C_m\) values and lower refolding rates. The present data have provided evidence that the disulfide, Cys\(^{141}\)-Cys\(^{163}\), plays a substantial role in sustaining the stability of the molecule and accelerating the refolding of denatured DsbC and contributes to the formation of a large uncharged surface of the V-shaped cleft (14) for substrate binding. Lack of the Cys\(^{141}\)-Cys\(^{163}\) disulfide, although it affects neither the export nor the processing, does decrease the solubility of the expressed mutants greatly so as to aggregate in the periplasm.

It is known that many overexpressed recombinant proteins are liable to form inclusion bodies in the cytoplasm, but mostly soluble if targeted in the periplasm (32, 33). There are a few reports on periplasmic inclusion bodies (33, 34). Using the export pathway directed by a His-tagged signal and optimal induction by IPTG at 0.01 mM recently established in this laboratory (21), wild-type DsbC has been overproduced as a properly processed and soluble periplasmic protein; however, the mutants with Cys replaced by Ser, although processed correctly, behave differently in terms of solubility and/or cellular distribution. Lowering temperature to 25 °C increased the export and processing efficiency but did not improve the solubility of overexpressed proteins in the periplasm (data not shown). The His-tagged signal sequence does not affect the correct processing of the modified signal, since all the mutants have the authentic N terminus. The aggregation of C98S, C141S, C163S, and C101S/C163S in the periplasm does not appear to be resulted from the proteolysis resistance, a driving force for the formation of inclusion bodies in many cases, since all the insoluble mutants are digested by proteinase K. The possibility that the His tag at the N terminus of the signals affected the folding of mutated molecules was also excluded since wild-type DsbC is expressed as an easily soluble protein in the periplasm using the same expression system and the mutation sites are distant from the signal in both sequence and three-dimensional structure (14). Thus, it is suggested that the low stability of mutants of C141S, C163S and C101S/C163S with low \(C_m\) values may contribute to the insolubility in the periplasm. It is also possible that the solubility of a periplasmic protein may relate to its conformation before it reaches the periplasm or during translocation. From the facts that DsbC, C98S/C101S, and C98S containing the intact Cys\(^{141}\)-Cys\(^{163}\) disulfide are soluble or partially soluble at least in the periplasm, and that C141S, C163S, and C101S/C163S with no Cys\(^{141}\)-Cys\(^{163}\) disulfide aggregate in the periplasm and refold in \textit{vitro} apparently slower than the formers do, it is rational to speculate that the formation of the nonactive site disulfide bond Cys\(^{141}\)-Cys\(^{163}\) is a key factor to stimulate further folding to a proper conformation. Failure of the formation of this disulfide may allow the folding intermediates to have their non-native hydrophobic surface exposed for a longer time and therefore more chance for incorrect interactions to form aggregates.

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Disulfide-dependent Folding and Export of *Escherichia coli* DsbC

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