The N-terminal Sequence (Residues 1–65) Is Essential for Dimerization, Activities, and Peptide Binding of Escherichia coli DsbC*

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The Dsb protein family in bacterial periplasm has been recently characterized to be responsible for the formation of native disulfide bonds of newly synthesized polypeptides (1). At least six proteins, DsbA, DsbB, DsbC, DsbD, DsbE, and DsbG, have been identified, and they work in conjunction with one another (2). DsbC is a soluble protein catalyzing the rearrangement of dethioleaved protein-disulfide oxidoreductase (TPOR) activity and neither isomerase nor oxidoreductase activity.

Limited proteolysis of DsbC with trypsin resulted in a compact and stable C-terminal fragment (residues 66–216), fDsbC, which retains the active site sequence, -Cys98-Gly-Tyr-Cys101—, and shows only minor differences in conformation compared with that of the intact molecule. The pK\text{a} of active site thiol and the K_{SS} with glutathione are very close to that of DsbC, respectively; however, fDsbC is inactive as an isomerase in catalyzing the formation of correct disulfide bonds in scrambled RNase A and reduced and reoxidized bovine pancreatic trypsin inhibitor and shows only 13% thiol-protein oxidoreductase activity (TPOR) of DsbC. In contrast to the trypsin inhibitor and shows only 13% thiol-protein oxidoreductase activity (TPOR) of DsbC. In contrast to the trypsin inhibitor, fDsbC exists as a monomer and has no chaperone activity in assisting the reactivation of denatured RNase A and denatured and reduced bovine pancreatic ribonuclease; sRNase, RNase A with scrambled disulfide bonds; mRNase, RNase A protein oxidoreductase; ANS, 8-anilino-1-naphthalenesulfonic acid; PAGE, polyacrylamide gel electrophoresis; mmDsbC and mmfDsbC, DsbC and fDsbC, respectively.

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was from Merck. All other chemicals were local products of analytical grade.

**Preparation**—The expression plasmid pDsbC containing the full-length DsbC precursor gene is a generous gift from Dr. Rudi Glockshuber ( Eidgenössische Technische Hochschule, Hönggerberg, Switzerland). It was prepared according to Missiaen et al. (15) and Chen et al. (8). α-Glycerolphosphate-3-phosphate dehydrogenase (GDPDH) from rabbit muscle (14) was kindly provided by J. Li and N. X. Zhang of this laboratory. Scrambled RNase A (sRNase) was prepared essentially according to Hillson et al. (15). S-Carboxymethylated RNase A (mRNase) was prepared by overnight incubation of RNase A at 1.8 mm with 1.30 mM iodoacetamide and 6 M GdnHCl in 0.4 M Tris-HCl buffer (pH 8.0) at room temperature and then modification with a 10-fold excess of iodoacetic acid in 1.5 M Tris-HCl with 6 M GdnHCl (pH 9) at room temperature for 1 h. The reaction mixture was adjusted to pH 4–4.5 with glacial acetic acid, dialyzed thoroughly against 0.02 M acetic acid at 4 °C, and lyophilized.

DsbC was prepared by limited digestion of DsbC with trypsin. DsbC at 1 mg/ml was incubated with trypsin at 0.01 mg/ml in 0.1 mM ammonium bicarbonate buffer (pH 8.1) at 37 °C for 2–3 h. The reaction mixture was then loaded on a Bio-Scale Q column (Bio-Rad) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) and eluted with a linear gradient of 0–0.2 M NaCl in 20 ml of the same buffer. The main peak was collected, dialyzed thoroughly against 50 mM NH₄HCO₃, at 4 °C, and lyophilized.

Thiol groups were determined by reaction products on 15% SDS-polyacrylamide gel electrophoresis (PAGE). The samples were analyzed by reverse phase FPLC on a PepRPC™ HR 5/5 column (Amersham Pharmacia Biotech) at room temperature with a gradient of 30–38% (v/v) acetonitrile in 20 ml of 0.1% trifluoroacetic acid.

**Activity Assay**—Activities of disulfide isomerase based on the isomerization of sRNase to the native enzyme and TPOR using the reduction of insulin with a linkage to glutathione reductase were assayed according to Lambert and Freedman (20), protein-disulfide oxidoreductase (PDOR) was determined based on the increases of fluorescence emission at 519 nm upon the reduction of intercatenary disulfide bonds of difluoresceinioicarbamyl-insulin (21). The effects of DsbC and DfDsbC on the disulfide-coupled refolding reaction of fully reduced BPTI were performed basically according to Zapun and Creighton (22). The refolding reaction mixture was incubated at 25 °C for 20 min before the addition of reduced BPTI to initiate the reaction in order to ensure the redox equilibrium in the active site of DsbC and DfDsbC under the used concentration of GSSG/GSH. The acid-trapped samples were analyzed by FPLC on a PepRPC™ HR 5/5 reverse phase column at room temperature with a gradient of 9–36% (v/v) acetonitrile in 50 ml of 0.1% trifluoroacetic acid.

**Denaturation and Renaturation of GAPDH—** Denaturation with GdnHCl and assisted reactivation of GAPDH upon dilution in the presence of DsbC and DfDsbC were carried out according to Chen et al. (8). Aggregation during refolding of denatured GAPDH was followed continuously at 25 °C by 90° light scattering at 488 nm in a Hitachi F-4010 spectrophotofluorimeter.

### RESULTS

**Limited Digestion of DsbC by Trypsin—** As shown in Fig. 1, by digestion of 1 mg/ml DsbC at 37 °C with trypsin at a weight ratio of 0.01, the DsbC band disappeared completely after 100 min, and after 3 h a fragment of 17 kDa was the only digestion product detectable on the gel. This component was purified through one-step anion exchange chromatography and named as DfDsbC.

**Identification of DfDsbC—** The first three amino acid residues at the N terminus of DfDsbC have been determined to be Met-Leu-Leu, indicating that DfDsbC results from the cleavage of DsbC between Lys⁶⁴ and Met⁶⁵ by trypsin (Scheme I). The mass of DfDsbC determined by mass spectrometry is 16,683 Da, in agreement with the calculated value of 16,687 Da for the fragment of 66–216.

**Oligomeric State—** Upon size exclusion chromatography, DsbC was eluted at the position corresponding to an apparent mass of 58 kDa, similar to the 67 kDa reported by Zapun et al. (3), and DfDsbC was eluted at 22 kDa, suggesting that the DsbC monomer is a dimer, the latter as a monomer. It should be noted that both of the apparent mass values are higher than the expected values of 46.9 and 16.7 kDa, respectively and the reduced forms of DsbC and DfDsbC, mmDsbC and mmfDsbC, showed exactly the same elution position as those of the respective oxidized forms, indicating that redox state and thiol modification do not affect the association properties. Thus,
mmDsbC is a dimer, and mmfDsbC is a monomer.

As shown in Fig. 2, the nondenaturing PAGE of the hybrid mixtures of DsbC/mmDsbC at all ratios contain three bands (Fig. 2, lanes 2–6) for the native protein, the heterodimer, and the modified protein, while the mixture of fDsbC/mmDsbC shows only two bands with no heterodimer between the two homodimers (lane 9). In addition, no band of heterodimeric hybrid appeared for DsbC/mmDsbC (lane 11) and DsbC/fDsbC (lane 12). The above finding indicates the dimeric state of DsbC and the monomeric state of fDsbC and mmfDsbC, in agreement with the results of gel filtration.

**Cysteine Thiol Groups of fDsbC—fDsbC freshly prepared, like DsbC, is in the oxidized form, since no thiol group was detected by Ellman’s assay when it was denatured with 6.4 M GdnHCl.**

DsbC and fDsbC, carboxymethylated at the N-terminal thiol in the active site sequence -Cys46-Gly-Tyr-Cys101-, show 0.8 and 0.9 thiol groups, respectively, after denaturation, suggesting that in DsbC, like in DsbC (8), only the N-terminal thiol group (Cys46) of the active site was alkylated in the native conformation, so that only Cys101 can be detected after denaturation. The other two non-active site thiol groups form a disulfide bond.

There is no significant change in reactivity of Cys46 in DsbC caused by removing the N-terminal segment (residues 1–65) as shown by the pKa values, 4.3 ± 0.2 (n = 3) for DsbC as compared with 4.1 ± 0.3 (n = 6) for DsbC (Fig. 3). As shown in Fig. 4, the equilibrium constant KSS for the formation of the accessible disulfide bond of fDsbC with glutathione is 205 μM, which is close to the KSS of 273 μM of DsbC (3), indicating that the disulfide bond of fDsbC at the active site is nearly as unstable as that of DsbC.

**Fluorescence and CD Spectra—**DsbC contains one Trp at position 140 and eight Tyr residues, whereas fDsbC keeps Trp140 but has only six Tyr residues. The intrinsic fluorescence spectra of fDsbC with excitation wavelengths at either 280 or 295 nm are similar to that of the intact molecule in terms of the emission maximum but with lowered emission intensity (Fig. 5, A and B). The ANS fluorescence spectra are nearly the same for DsbC and fDsbC (Fig. 5C). The CD spectrum of fDsbC is also similar to that of DsbC (Fig. 5D).

For oxidized and reduced fDsbC, like DsbC (3) but unlike DsbA (23), there is no difference between the intrinsic fluorescence spectra with excitation wavelength at either 280 or 295 nm, ANS fluorescence spectra, and CD spectra (data not shown), indicating that the oxidized and reduced fDsbC have essentially the same fold.
The fluorescence spectra; A with excitation at 280 nm (GSH. Filled reverse phase FPLC. equilibrium reaction mixtures were acid-quenched and analyzed by two concentrations of GSSG and varying amounts of GSH in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM EDTA at 25 °C for 20 min. The active site disulfide bond of fDsbC and DsbC with glutathione.Curve 1, fDsbC; Curve 2, DsbC; Curve 3, mmDsbC; Curve 4, mmfDsbC.

Table II shows the relative intensity of the three bands for the native protein, the heterodimer, and the inactive modified protein, the TPOR activity, and the isomerase activity of the hybrid mixtures with different ratios of DsbC/mmDsbC in Fig. 2. The native species, DsbC, is fully active, and the modified mmDsbC is inactive. It is noted that the experimentally determined TPOR activities of hybrid mixtures agree well with the sums of the relative intensity of the native and the half-intensity of the heterodimer bands. The isomerase activities experimentally determined are very close to the corresponding values of the relative band intensity of the native species. The above finding suggests that the heterodimer of DsbC/mmDsbC shows little (if any) isomerase activity but almost 50% TPOR activity of DsbC; i.e. the DsbC subunit in the heterodimer displays full TPOR activity but little (if any) isomerase activity. The isomerase activities of DsbC have been determined in the presence of a 20-fold molar excess of DsbC, the aggregation of GAPDH in the presence of DsbC increases from 6 to 30% as the molar ratio of DsbC to GAPDH increases to 25.

Effects of fDsbC on Reactivation and Aggregation of Denatured GAPDH—Reactivation of denatured GAPDH was used to examine the chaperone activity of DsbC independent to its thiol-protein oxidoreductase activity (5). As shown in Fig. 7A, reactivation of GAPDH in the presence of DsbC increases from 6 to 30% as the molar ratio of DsbC to GAPDH increases to 25. However, fDsbC at the same range of molar ratios shows no effect on the reactivation of the denatured GAPDH. In the presence of a 20-fold molar excess of DsbC, the aggregation of

Table I

|          | Km (μM) | kcat (min⁻¹) | kcat/Km (μM⁻¹ min⁻¹) |
|----------|---------|--------------|-----------------------|
| DsbC     | 5.72 ± 0.24 | 1.875 ± 0.0154 | 0.328                 |
| fDsbC    | 14.76 ± 0.41 | 0.232 ± 0.0013 | 0.0157                |
| fDsbC/DsbC | 2.58 | 12.4% | 4.8% |

Effects of fDsbC and DsbC on the refolding of reduced BPTI. Reactions were initiated by the addition of fully reduced BPTI to a final concentration of 10 μM into 0.1 M Tris buffer (pH 7.5) with 0.2 M KCl, 1 mM EDTA, 0.5 mM GSSG, and 2 mM GSH in the absence (A, B) or presence of 8 μM DsbC (C, D) or 8 μM fDsbC (A, D) at 25 °C. The aliquot was withdrawn for acidification at various times as indicated. The native (open symbols) and the reduced BPTI (solid symbols) were separated and quantified by reverse phase FPLC.

Fig. 6. Effects of fDsbC and DsbC on the refolding of reduced BPTI. Reactions were initiated by the addition of fully reduced BPTI to a final concentration of 10 μM into 0.1 M Tris buffer (pH 7.5) with 0.2 M KCl, 1 mM EDTA, 0.5 mM GSSG, and 2 mM GSH in the absence (A, B) or presence of 8 μM DsbC (C, D) or 8 μM fDsbC (A, D) at 25 °C. The aliquot was withdrawn for acidification at various times as indicated. The native (open symbols) and the reduced BPTI (solid symbols) were separated and quantified by reverse phase FPLC.
denatured GAPDH during refolding decreases significantly in both rate and extent, but fDsbC at the same concentration has no effect (Fig. 7B).

**DISCUSSION**

Compact Structure of fDsbC—Although there are 21 potential sites for trypsin cleavage in DsbC, as shown in Scheme I, the protein is only cleaved at a limited number of sites under the employed conditions with the site between Lys65 and Met66, one of the most liable to be cleaved. All of the 16 sites within the DsbC fragment seem to be unaffected during the first 3 h of digestion at 37 °C even with 1:10 (weight ratio) trypsin or prolonged digestion time of 20 h with 1:100 trypsin at 37 °C. The resistance toward trypsin proteolysis of the C-terminal fragment suggests a compact structure of this part of the DsbC molecule. The peptide segment around the nick site between Lys65 and Met66 appears to be a flexible and exposed loop or a mobile linker region between domains (11). The nick site is indeed located within the movable hinged linker α-helix, which connects the N-terminal domain (residues 1–61) and the C-terminal domain (residues 78–216) with a thioredoxin fold (9) and is most accessible for trypsin attack. The fragment fDsbC is thus just the C-terminal thioredoxin domain with 12 residues in the linker at the N terminus.

The N-terminal domain, consisting of six-stranded anti-parallel β-sheets (9), is obviously much less compact than the C-terminal region for its greater susceptibility to digestion, since in the first 50 min of digestion two additional weak bands with a mass of about 21 kDa (cleaved between Lys28 and Thr29) and 19 kDa (cleaved between Lys44 and His45) appeared in addition to the band of the intact molecules and the main band with a mass of 17 kDa (cleaved between Lys65 and Met66) and...
is digested to small pieces during trypsin treatment.

**fDsbC Is Monomeric in Contrast to the Dimeric DsbC**—By size exclusion chromatography and non-denaturing electrophoresis of hybrid mixtures, it is concluded that fDsbC is monomeric. The fact that fDsbC retains the general conformation as in the intact molecule led us to suggest that the N-terminal fragment of 65 residues is responsible for the dimerization of DsbC subunits, and this is now confirmed by the crystal structure (9). The two subunits of DsbC form a V-shaped molecule with each arm consisting of two separate domains connected by a hinged linker helix (see Scheme II). The N-terminal domains (residues 1–61) from each monomer form a pair of six-stranded anti-parallel β-sheets that form the dimer interface at the base of the V-shaped molecule. The N-terminal domain is thus named the dimerization domain. It becomes very clear that removal of the dimerization domain results in fDsbC as two separate monomeric C-terminal thioredoxin domains.

In non-denaturing PAGE of a hybrid mixture of DsbC/mmDsbC at a ratio of 1:1, the relative proportion of the native protein, the heterodimer, and the inactive modified protein is not 1:2:1 as would be expected from random association of the subunits but actually approximately 1:1:1. It is interesting to find that a rerun of non-denaturing PAGE of the recovered heterodimeric protein of DsbC/mmDsbC from the gel shows again three bands with a ratio of approximately 1:1:1 (data not shown). The above result indicates a lower affinity to form heterodimers compared with that of homodimers and an equilibrium between the heterodimer and the homodimers. According to the crystal structure, the dimerization is essentially via the hydrogen bonds between the β-sheets of the N-terminal domains and the two C-terminal thioredoxin domains with the active sites are normally 38 Å apart across the cleft. In addition, the linker helix is sufficiently long that the N-terminal association domain and the C-terminal catalytic domain within a monomer also have minimal interaction. It is hard to understand why the modification at the active site of one subunit affects the dimerization with a normal subunit unless the moderate perturbed conformation of the modified subunit is responsible. We also found that the heterodimer appeared from a mixture of DsbC and mmDsbC under non-denaturing conditions, indicating that native DsbC and mmDsbC also exist in equilibrium of dissociation and association (data not shown). However, the process under non-denaturing conditions is very slow and takes about 24 h to reach equilibrium.

**Enzyme and Chaperone Activities**—fDsbC, as a monomer, shows no isomerase activity in catalyzing the formation of native molecules either from sRNase or from reduced BPTI but retains 13.5% TPOR activity and part of the oxidase activity of homodimeric DsbC.

It is suggested that the combination of the two basic structural features, i.e. a large uncharged surface (40 × 40 × 25 Å) consisting of the cleft of the V for substrate binding and a thiolate active site, account for its isomerase activity. The broad uncharged cleft in DsbC molecule is sufficient to allow the binding of target protein and may be involved in both the chaperone and isomerase activities (9). Moreover, the hinged linker helix may provide sufficient flexibility to allow the binding of different sized substrates (9).

Since fDsbC has an intact active site with unchanged chemical properties (the protruded Cys98, pK_a and redox constant K_SS) and a slightly perturbed molecular conformation, the absence of the large uncharged surface within the V-shaped dimeric molecule resulting from the removal of the N-terminal dimerization domains very likely leads to the absence of isomerase activity. In this connection, the K_m of fDsbC for insulin is 2.58-fold higher than that of DsbC, indicating a poorer substrate binding with fDsbC than with DsbC. The fact that the DsbC monomer in the heterodimer of DsbC/mmDsbC shows no isomerase activity but full TPOR activity of DsbC suggests that both of the active sites of dimeric DsbC may be necessary for its isomerase activity and that the monomeric state is sufficient for its TPOR activity. Our present data provide an experimental demonstration of the speculation by Zapun et al. (3) that in the catalyzed isomerization of mispaired disulfides of a scrambled protein with no free thiols, the simultaneous break of the other disulfide may be needed, so that a different disulfide can be formed via disulfide interchange. Similar to PDI, the two active sites with one in each domain of DsbC are obviously favorable and probably necessary to catalyze the isomerization of scrambled substrate. The catalyzed reduction of a disulfide only needs one active site of the enzyme to function. In this respect, it is speculated that in the recycling of DsbC in cells the reduction of the disulfide in each domain of DsbC by DsbD may be independent. In the determination of reaction kinetics of DsbC with glutathione, Zapun et al. (3) proposed that the two active sites of DsbC function independently, since no indication of interactions between the two monomers of the DsbC dimer was found. Meanwhile, the sufficient area for substrate binding is also necessary for TPOR and oxidase activities, since fDsbC lacking the N-terminal domain has much lower activities. The possibility is not excluded that the conformational change in modified subunit could be responsible for the activities of the heterodimer. It is also to be recalled that in contrast to the dimeric DsbC and PDI with substantial isomerase activity, thioredoxin is monomeric and shows little isomerase activity (24). DsbA is also monomeric but has an additional helical insert, which, together with the thioredoxin domain, provides a much more extended hydrophobic surface than that of thioredoxin for substrate binding, and therefore DsbA shows low isomerase activity of about 5% of that PDI (25) and 20% of DsbC (3). In this connection, it is known that the active sites of some enzymes are shared between different subunits (26, 27). For DsbA and the monomeric domain a of PDI with 14% isomerase activity (28) of PDI, their small size may allow two enzyme molecules to attack two disulfide bonds of a substrate simultaneously.

Since the chaperone and PDOR activities of DsbC can be inhibited by sRNase and mRNase at high concentrations but not by small peptides, it has been suggested that DsbC has an extended surface for peptide binding so that only a relatively large unfolded peptide is able to compete with the substrate or the target folding intermediate for binding to DsbC (8). fDsbC is no longer active as a chaperone on the reactivation and prevention of aggregation of denatured GAPDH, and its PDOR...
activity is not inhibited by mRNase, suggesting that fDsbC has lost the ability for large peptide binding and consequently the activity as a chaperone. This has now also been supported by the three-dimensional structure (9). In contrast to mRNase, sRNase decreases PDOR activity of DsbC and fDsbC at very low concentrations, since DTT present in the determination of PDOR activity may reduce the disulfide of sRNase and lead to disulfide exchange with DsbC and fDsbC, and results in the inhibition of the PDOR activity. Many chaperones have ring structures to form a peptide binding site at the end of the ring contributed by each subunit (29); e.g. GroEL has two seven-member rings, the eukaryotic cytosolic chaperonin, CCT, and archaebacteria thermosome have two eight-member rings, and archaebacteria TF55 has two rings of nine subunits. In contrast, in other chaperones, like dimeric PDI (30) and DsbC, the multidomain structure seems to contribute to form an extended site for substrate binding.

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