

Dimerization by Domain Hybridization Bestows Chaperone and Isomerase Activities*

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Thioredoxin, DsbA, the N-terminal active-site domain a and the non-active-site domain b of protein-disulfide isomerase are all monomeric with a thioredoxin fold, and each exhibits low or no isomerase and chaperone activity. We have linked the N terminus of the above four monomers, individually, to the C terminus of the N-terminal domain of DsbC via the flexible linker helix of the latter to produce four domain hybrids, DsbCn-Trx, DsbCn-DsbA, DsbCn-PDIa, and DsbCn-PDIb. These four hybrid proteins form homodimers, and except for DsbCn-PDIb they exhibit new or greatly elevated isomerase as well as chaperone activity. Three-dimensional structure prediction indicates that all the four domain hybrids adopt DsbC-like V-shaped structure with a broad uncharged cleft between the two arms for binding of non-native protein folding intermediates. The results provide strong evidence that dimerization creates chaperone and isomerase activity for monomeric thiol-protein oxidases or reductases, and suggesting a pathway for proteins to acquire new functions and/or higher biological efficiency during evolution.

Many proteins, such as secretory proteins (antibodies, some peptide hormones) and membrane proteins (receptors, channel proteins), contain disulfide bonds, which play an essential role in stabilizing the tertiary and quaternary structures of these molecules. The formation of native disulfide bonds (including disulfide isomerization) is a key step in protein folding and is usually catalyzed by thiol-protein oxidoreductases, protein-disulfide isomerase (PDI)¹ in eukaryotes, and Dsb proteins in prokaryotes. So far at least six members of the Dsb family, DsbA, DsbB, DsbC, DsbD, DsbE, and DsbG, have been identified. In recent years PDI (1–4), DsbC (5), and DsbG (6) have been characterized to exhibit both disulfide isomerase and chaperone activity. The thiol-protein oxidoreductases contain

thioredoxin (Trx) fold with one or more motif(s) of -CX₂C- as active site(s). PDI is a homodimeric molecule mainly located within the endoplasmic reticulum, and each subunit is composed of four successive Trx-fold domains (**a-b-b'-a'**) and a C-terminal tail (7). It is known that **a** and **a'** are homologous, and each has a -CGHC- motif as an active site. However, **b** and **b'**, without such an active site motif, are homologous with each other but not with the **a** domain. DsbC, located in the periplasm, is a prokaryotic counterpart of PDI and has been shown by crystal structure analysis (8) to be a V-shaped homodimer with each subunit forming an arm of the V. The N-terminal domain (1–61) of the subunit is linked via an α -helix-hinged linker (62–77) to the C-terminal Trx-domain (DsbCc, 78–216) with a -CGYC- motif as an active site. The N-terminal domain from each monomer forms the dimer interface at the base of the V through β -sheet hydrogen bonds. The broad uncharged cleft with a large hydrophobic surface within the V has been suggested to be the site for peptide binding and therefore it plays a role in both the chaperone and the foldase activity of DsbC (8). Biochemical data (9) reveal that deletion of the first 65 residues at the N terminus prevents the dimerization of the subunits and the gain of chaperone activity. These observations are consistent with crystal structural analysis. A point mutation at the conserved Gly-49 has been reported to cause DsbC to exist as a monomer instead of a dimer and to convert it from an isomerase to an oxidase (10). Therefore the dimeric structure of DsbC appears to be a critical factor for achieving its multifunctional activities, namely functions as both isomerase and chaperone.

Trx, DsbA, and the **a** domain of PDI are all monomeric proteins. They exhibit low or no isomerase and chaperone activity, even though they contain the Trx fold with -CX₂C- motif. In this report we have expressed and purified four domain hybrids, namely, DsbCn-Trx, DsbCn-DsbA, DsbCn-PDIa, and DsbCn-PDIb. In these hybrids the N terminus of Trx, DsbA, the **a** and the **b** domain of PDI has been separately linked to the C terminus of the association domain by the flexible linker helix of DsbC (Scheme I). We investigated whether the hybrids could form a dimeric structure, and if they do, is there a gain of function for these four Trx promoters. The results reveal that hybridization has indeed produced homodimeric structure for the four hybrids and the hybrids which contain Trx, DsbA, and the **a** domain of PDI are endowed with chaperone and isomerase activity. Our findings reveal a strategy by which biological molecules could achieve new functions and gain higher efficiency through evolution process.

EXPERIMENTAL PROCEDURES

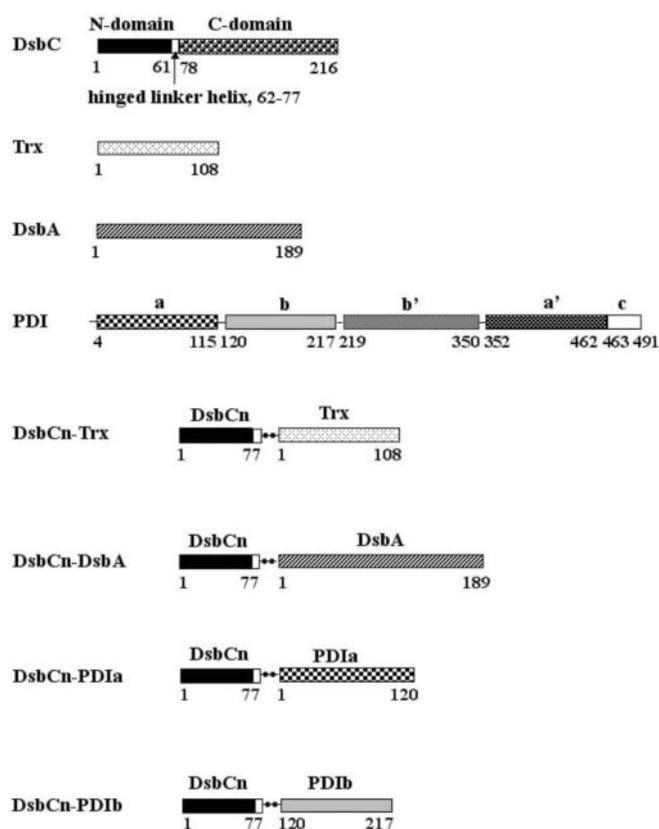
Materials—The plasmid pDsbC, containing the full-length coding sequence of *E. coli* DsbC precursor, was generously given by Dr. R. Glockshuber, Eidgenössische Technische Hochschule Honggerberg, Switzerland. The plasmids, pTrx containing the full-length coding sequence of *Escherichia coli* Trx and pUCBM21-DsbA containing the

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¹ The abbreviations used are: PDI, protein-disulfide isomerase; Trx, thioredoxin; DsbCn, N-terminal domain with the hinged linker helix of DsbC (residues 1–77); DsbCc, C-terminal domain of DsbC (residues 78–216); PDIa, N-terminal active site domain of protein-disulfide isomerase; PDIb, N-terminal non-active site domain of protein-disulfide isomerase; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol.



SCHEME I. Constructions of domain hybrids. The fragment (1–77) of DsbC, DsbCn (the N-terminal domain, 1–61, and the hinged linker helix, 62–77), was linked to the N terminus of Trx (1–108), DsbA (1–189), the **a** domain (1–120) and the **b** domain (120–217) of human PDI (7), to construct the hybrids of DsbCn-Trx, DsbCn-DsbA, DsbCn-PDIa, and DsbCn-PDIb respectively. The *black dots* following DsbCn represent the two extra residues, Val-Asp, added because of *Sall* sites in plasmid constructions.

full-length coding sequence of *E. coli* DsbA precursor, were kindly provided by Professor S. L. Yang, Shanghai Research Center of Biotechnology, Chinese Academy of Sciences. The plasmid pBR322-PDI, containing the full-length cDNA of human PDI, is a generous gift from Prof. K. Kivirikko, University of Oulu, Finland. DsbC and DsbA were prepared according to Liu *et al.* (11), and Trx to Sun *et al.* (12). PDI from bovine liver (13), D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle (14) and *E. coli* DsbC (5) were kindly provided by J. Li, G. P. Ren, and C. J. Huang, respectively in our group. Expression vector of pQE-70, *E. coli* DH5 α and M15[REP4] were from Qiagen. Bovine serum albumin (98–99% albumin; fraction V), glyceraldehyde-3-phosphate, guanidine hydrochloride (GdnHCl), imidazole, NAD⁺ (97%), scrambled ribonuclease A, and yeast RNA were Sigma products. Dithiothreitol (DTT) and isopropyl β -D-thiogalactopyranoside were from Serva, restriction enzymes and Tris from Promega and molecular mass markers from Bio-Rad. Other chemicals were local products of analytical grade.

Construction of Expression Plasmids—The domain hybrids of DsbCn linked respectively to the N terminus of Trx, DsbA, the **a** and the **b** domain of human PDI by the hinged linker helix of DsbC were constructed as shown in Scheme I.

The genes coding for DsbCn, Trx, DsbA, PDIa, and PDIb were amplified from pDsbC, pTrx, pUCBM21-DsbA, and pBR322-PDI, respectively using primers as listed in Table I for PCR: P1 and P2 for DsbCn, P3 and P4 for Trx, P5 and P6 for DsbA, P7 and P8 for PDIa, and P9 and P10 for PDIb. The PCR fragment for DsbCn was subcloned into pUC18 at *Bam*HI and *Sall* sites to construct pUC-DsbCn. The PCR fragments for Trx, DsbA, PDIa, and PDIb were subcloned into pUC18 at *Sall* and *Hind*III sites to construct pUC-Trx, pUC-DsbA, pUC-PDIa, and pUC-PDIb, respectively.

The DsbCn sequence with the ends of *Bam*HI and *Sall* excised from pUC-DsbCn was inserted into pUC-Trx, pUC-DsbA, pUC-PDIa, and pUC-PDIb to construct pUC-DsbCn-Trx, pUC-DsbCn-DsbA, pUC-DsbCn-PDIa, and pUC-DsbCn-PDIb, respectively. There are two extra

TABLE I
Primers designed for constructions of domain hybrids

Primer	Sequence
P1	5'-CGGGATCCATGAAGAAAGGTTTTATG-3'
P2	5'-GCGTCGACCTCTTTTTCAAGCGCA-3'
P3	5'-GCGTCGACAGCGATAAAATTATTCACC-3'
P4	5'-CCAAGCTTACGCCAGGTTAGCGTC-3'
P5	5'-GCGTCGACGCGCAGTATGAAGATG-3'
P6	5'-CCAAGCTTATTTTTCTCGGAC-3'
P7	5'-GCGTCGACGACGCCCCCGAGGAG-3'
P8	5'-CCAAGCTTAGGCAGCCCGGCCCG-3'
P9	5' GCGTCGACGCCACCACCCTGCCTGACG-3'
P10	5' CCCAAGCTTACAGCTGGTTGTGTTTGATAAAG-3'

amino acid residues, Val-Asp, resulting from the *Sall* site at the domain junctions. The full-length coding sequences of DsbCn-Trx, DsbCn-DsbA, DsbCn-PDIa, and DsbCn-PDIb with the ends of *Bam*HI and *Hind*III were excised from their corresponding pUC plasmids and then subcloned into pQE-70 to construct corresponding pQE-DsbCn-Trx, pQE-DsbCn-DsbA, pQE-DsbCn-PDIa, and pQE-DsbCn-PDIb, respectively. The resulting constructs were identified by diagnostic PCR and DNA sequencing.

Expression and Purification of Proteins—Transformed M15[REP4] cells were grown in LB media with 100 μ g/ml ampicillin and 25 μ g/ml kanamycin. The overnight-cultured seed cells were diluted 100-fold and incubated for another 4 h followed by overnight induction at 25 $^{\circ}$ C using isopropyl β -D-thiogalactopyranoside at 0.01 mM except 0.05 mM for DsbCn-PDIa. The expression and processing of target proteins were examined by sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-PAGE) (11).

Target proteins released from the periplasm of harvested cells by osmotic shock were dialyzed overnight against 50 mM Tris-HCl buffer (pH 7.8) for DsbCn-Trx, DsbCn-DsbA, and DsbCn-PDIb and 10 mM phosphate buffer (pH 6.8) for DsbCn-PDIa. After centrifugation at 15,000 \times *g* at 4 $^{\circ}$ C for 20 min the supernatant was loaded onto a Q-Sepharose Fast Flow column (Amersham Biosciences) and eluted with a gradient of 0–0.4 M NaCl in the same buffer. The main peak was collected, desalted, and stored with 10% glycerol at –80 $^{\circ}$ C. The purified proteins showed one band on SDS-PAGE.

Concentration and Spectrum Analysis—Protein concentrations were determined by the method of Bradford (15) with bovine serum albumin as a standard. For the convenience of comparison, homotetrameric GAPDH, homodimeric DsbC, PDI, and all domain hybrids were taken as protomers in the calculation of molar ratios.

CD spectra from 200 to 250 nm were determined with a Jasco 500 spectropolarimeter at 25 $^{\circ}$ C, and the amount of α -helix was calculated according to Pownall *et al.* (16).

Assays of Enzyme and Chaperone Activities—Disulfide isomerase activity was assayed by the method of Lambert and Freedman (13) according to the re-activation of scrambled ribonuclease A. The specific activity is expressed as unit/ μ mol instead of unit/g.

Thiol protein reductase activity was assayed by measuring the turbidity increase at 650 nm due to insulin reduction (17). The activity is expressed as the ratio of the slope of a linear part of the turbidity curve to the lag time (18).

The reactivation of GdnHCl-denatured GAPDH upon dilution in the presence of domain hybrids at difference concentrations was carried out according to Chen *et al.* (5) to determine chaperone activity of the hybrids.

Three-dimensional Structure Prediction—Homologous sequence searching was performed throughout the two data bases, PDB (19) and Swiss-Prot, using BLASTP (20) and FASTA (21). Only sequences respectively homologous to the two domains in hybrids were found. The three-dimensional structures of DsbC (1EEJ), Trx (2TRX), DsbA (1DSB), PDIa (1MEK), and PDIb (1BJX) are all available in PDB. Therefore it is possible to predict the three-dimensional structures of the four hybrids by packing of the two component domains, respectively.

Crystal structure analysis of DsbC (8) has shown that in each subunit the N-terminal domain is linked to DsbCc by a flexible hinged helix, which is packed to DsbCc. The three-dimensional structural alignment of Trx, DsbA, PDIa, and PDIb separately with DsbCc was carried out using TOPP program (22), and the results showed that the major subdomains, consisting of 4, 5, or 6 β -strands packed with α -helices at two sides, are structurally well aligned, ignoring minor subdomains consisting of varying number of α -helices. All the five domains show similar three-dimensional structures, and therefore the three-dimensional structure of DsbC was taken as a template for the four

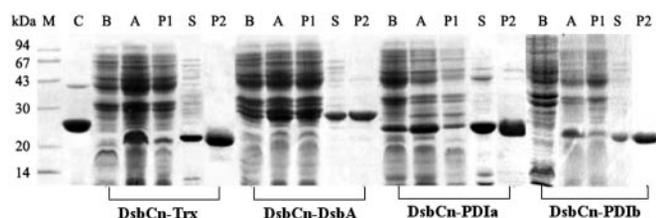


FIG. 1. SDS-PAGE (15%) profile of domain hybrids. *M*, molecular mass markers; *C*, purified DsbC; total cellular proteins of *E. coli* M15 bearing pQE-DsbCn-Trx, pQE-DsbCn-DsbA, pQE-DsbCn-PDIa, and pQE-DsbCn-PDIb before (*B*) and after (*A*) induction with isopropyl β -D-thiogalactopyranoside, respectively; pellet (*P1*) and supernatant (*S*) after osmotic shock; *P2*, purified protein.

hybrids. By fitting the three-dimensional structures of Trx, DsbA, PDIa, and PDIb in the hybrids to DsbC, the results show that each domain can pack to the linker helix in a similar way as that reported for DsbC. However, the position of the N-terminal segment of Trx, DsbA, PDIa, and PDIb needed to be relocated, so that the hydrophobic core on one side of the β -sheet became opened and the linker helix was packed onto the core. Based on the above information, three-dimensional structures of the hybrids were constructed using MODELLER (23).

RESULTS

Expression and Processing of the Recombinant Domain Hybrids—Like DsbC, all four domain hybrids of DsbCn-Trx, DsbCn-DsbA, DsbCn-PDIa, and DsbCn-PDIb were overexpressed as 20–50% of the total cellular proteins and transposed into the periplasm as soluble and correctly processed proteins with molecular mass of 20, 29, 22, and 19 kDa respectively as expected (Fig. 1).

DsbCn-Trx, DsbCn-DsbA, DsbCn-PDIa, and DsbCn-PDIb Are Homodimers—As shown in Fig. 2, the data from size-exclusion chromatography revealed that DsbC, DsbCn-Trx, DsbCn-DsbA, DsbCn-PDIa, and DsbCn-PDIb was eluted as a single peak at position corresponding to apparent molecular mass of 40, 37, 60, 38, and 50 kDa, respectively, indicating that all four hybrids, like DsbC, are homodimers in accord with the theoretical molecular mass (Table II).

Conformation of the Domain Hybrids—As shown in Fig. 3, the CD spectra of DsbCn-Trx, DsbCn-DsbA, and DsbCn-PDIa were similar to that of DsbC in both the shape and the ellipticities at 208 and 222 nm with α -helix content of 44, 45, and 42% respectively compared with 37% for DsbC. All the α -helix contents so determined are in reasonably agreement with the calculated values from the three-dimensional structural analysis (Table II). The CD spectrum of DsbCn-PDIb is somehow deviated from that of DsbC, but the α -helix content of 32% is also close to the calculated value.

Isomerase and Chaperone Activity of the Domain Hybrids—Compared with monomeric Trx, DsbA, and PDIa, the hybrids with DsbCn show greatly elevated isomerase activity as shown in Table III. The Trx had little, if any, isomerase activity but DsbCn-Trx showed significant activity, about 33% of the DsbC activity. The DsbA is primarily an oxidase showing low isomerase activity of about 10% of that of DsbC, but when linked to DsbCn, the hybrid exhibits a 5-fold increase in isomerase activity. Isolated PDIa, although being one of the two active site domains of PDI, showed no isomerase activity (29). However, DsbCn-PDIa displayed an enormous increase in isomerase activity, about 1.7-fold higher than that of DsbC.

As shown in Fig. 4 and Table III, dimerization does not alter significantly the reductase activity of the hybrids, which showed much lower reductase activity than that of either Trx or DsbC.

Fig. 5 shows that Trx failed to assist the reactivation of denatured GAPDH even at a high molar ratio to GAPDH of 35, and DsbA possessed a relatively low capacity to assist the

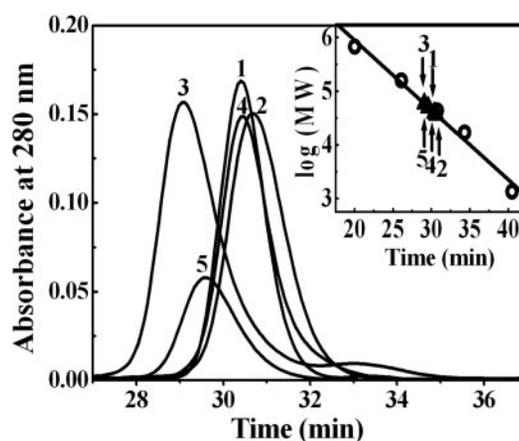


FIG. 2. Size-exclusion chromatography of domain hybrids. Chromatography was performed on a Superdex 200 HR 10/30 column (Amersham Biosciences) using 50 mM phosphate, pH 6.8, containing 150 mM NaCl. Curve 1, DsbC; curve 2, DsbCn-Trx; curve 3, DsbCn-DsbA; curve 4, DsbCn-PDIa; and curve 5, DsbCn-PDIb. Molecular mass markers (Bio-Rad) shown in the inset are: bovine thyroglobulin, 670 kDa; bovine γ -globulin, 158 kDa; chicken ovalbumin, 44 kDa; equine myoglobin, 17 kDa; and vitamin B₁₂, 1.35 kDa.

reactivation process. Nevertheless, the three hybrids, DsbCn-Trx, DsbCn-DsbA, and DsbCn-PDIa displayed a pronounced increase in chaperone activity in promoting the reactivation of denatured GAPDH. Compared with DsbC, which assisted the reactivation of GAPDH from 5 to 40% at a molar ratio to GAPDH of 20, DsbCn-DsbA and DsbCn-PDIa assisted the reactivation of GAPDH to a similar extent but at higher molar ratios, 30 for DsbCn-DsbA and 40 for DsbCn-PDIa. However, DsbCn-Trx showed even higher chaperone activity, it increases the reactivation of GAPDH to a maximum of 50% at a molar ratio of 30. The DsbCn-PDIb assisted the reactivation of GAPDH to a maximum of 18% at a molar ratio of 50, thus, it possesses rather low chaperone activity.

Three-dimensional Structures of the Domain Hybrids—Three-dimensional structures of the four domain hybrids were predicted with satisfactory stereo-chemical qualities. The Ramachandran plot by PROCHECK (30) displayed that more than 90% of the residues were distributed in the most favored region. Taking DsbCn-Trx as an example (see Fig. 6A), the superposition of the predicted structure of DsbCn-Trx on the crystal structure of DsbC is quite good. The hydrophobic residues on the linker helix, Leu-67, Leu-68, Leu-71, and Leu-74, make contacts with hydrophobic residues of Ile-23, Val-25, Phe-27, Leu-58, Leu-79, and Phe-81 on the β -sheet of Trx, and these interactions ensure the integrity of the whole structure. As the four hybrids have been characterized to be dimeric by biochemical methods, a DsbC-like V-shaped homodimer structure was suggested for all the hybrids with DsbCn-Trx as an example shown in Fig. 6B. By superposition of the two dimeric molecules of DsbC and DsbCn-Trx it was found that the surface of DsbCn-Trx is also dominated by a large cleft separating the active sites as in the DsbC molecule (Fig. 7). The dimension and the depth of the cleft are approximately the same as that found in DsbC, and the inner surface of DsbCn-Trx cleft is comprised of 49 hydrophobic or uncharged residues. It is noticed that the arginine residues found inside the cleft from each monomer were almost electrically neutral due to stereochemical characteristics. The broad uncharged cleft may account for peptide binding and chaperone activity, like that found in the DsbC molecule.

DISCUSSION

Molecular chaperones participate in a wide range of vital cellular processes. The chaperone machinery functions through

TABLE II
Molecular mass and α -helix content of domain hybrids

Protein	Molecular mass		α -helix content		
	Theoretical	Determined ^a	Calculated ^b	Determined ^c	
		<i>kDa</i>		%	
DsbC	23.415	40 \pm 1	36.9	37	
DsbCn-Trx	20.051	37 \pm 3	39.6	44	
DsbCn-DsbA	29.475	60 \pm 3	50.8	45	
DsbCn-PDIa	21.595	38 \pm 1	35.2	42	
DsbCn-PDIb	19.117	50 \pm 1	36.2	32	

^a Data from size-exclusion chromatography (Fig. 2) are expressed as mean \pm S.D. ($n = 3$).

^b Data calculated from the three-dimensional structure of DsbC (8), Trx (24), DsbA (25), PDIa (26), and PDIb (27) respectively.

^c Data calculated from the CD spectrum (Fig. 3) according to Pownall et al. (16).

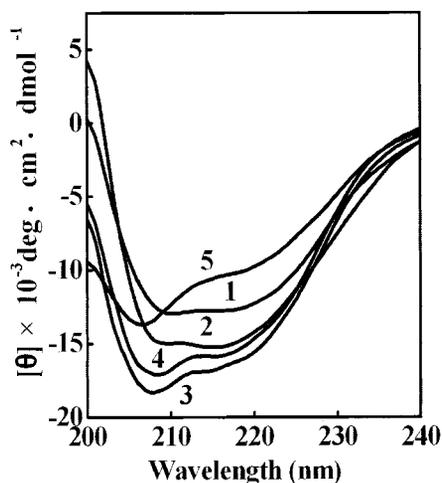


FIG. 3. CD spectra of domain hybrids. Curve 1, DsbC; curve 2, DsbCn-Trx; curve 3, DsbCn-DsbA; curve 4, DsbCn-PDIa; and curve 5, DsbCn-PDIb.

recognizing and binding to non-native conformational folding intermediates, protecting them from aggregation and promoting further folding, assembly, disassembly, and translocation, and mediating degradation of misfolded proteins. The structure of chaperones generally contains a peptide-binding site or surface, which is usually hydrophobic and very often located at an opening or inside of a cavity, like in GroEL molecule (32); or on a depression, like in Sis1 (33, 34); or on a few patches enclosing a central space, like in palm-shaped prefoldin (35). These structural characteristics provide chaperones with hydrophobic interactions with folding intermediates, and with a space to accommodate the intermediate. For chaperone DsbC, a broad cleft consisting largely of uncharged or hydrophobic residues between the two arms of the V-shaped molecule is believed to be the binding site for the unfolded peptide (8). Monomerization of DsbC, by either removing the association domain (9) or disrupting the dimerization interface resulted from a point mutation (10), eliminates its chaperone activity as well as its isomerase activity. Thus, dimerization of DsbC appears to play a vital role for it to function as both a chaperone and an isomerase. Trx (a thiol protein reductase), DsbA (an oxidase), PDIa (the N-terminal active site domain of PDI) and PDIb (the N-terminal non-active site domain of PDI) are all monomers with Trx fold and show low or no isomerase and chaperone activity. We have now demonstrated that fusion of Trx, DsbA, and PDIa to the association domain of DsbC not only induces the hybrids to form a homodimer, but also confers tremendously increased chaperone activity and isomerase activity. These results provide strong evidence that dimerization creates chaperone activity and isomerase activity for monomeric thiol protein oxidases and reductases. As PDIb does not contain the -CXYC- motif the hybrid of DsbCn-PDIb is a dimeric molecule but possesses only new chaperone activity.

The hybrid models constructed by three-dimensional structure prediction have provided structural bases to explain the experimental results obtained by biochemical methods in this report. Although the contacts between the linker helix and the C-terminal domain in hybrids are no longer identical with those in the DsbC molecule, the four hybrids, possessing a global packing with good superposition with DsbC molecule, all adopt very similar V-shaped structure as that of DsbC dimer under normal conditions. The size and the depth of the cleft between the two arms of the V shaped molecule in DsbCn-Trx is closely similar to that in native DsbC. There are 49 hydrophobic or uncharged residues on the inner surface of DsbCn-Trx cleft, which is similar to the 46 hydrophobic or uncharged residues found in the inner surface of the cleft of DsbC. In addition, the α -helix content determined by CD in each hybrid is in reasonable agreement with the calculated value from the three-dimensional structural analysis. These data indicate that hybridization has no significant effect on the secondary structure of the domains in hybrids. Another example is the yeast chaperone, Hsp40 protein Sis1, which also exists as a dimeric V- or U-shaped molecule. The crystal structure of Sis1 (171–352) showed that its C-terminal fragment remains as a dimer and has a U-shaped architecture with a large cleft formed between the two elongated monomers, and a large hydrophobic depression of 5 Å deep on the surface of the cleft. This hydrophobic depression has been proposed to be the peptide-binding site for Sis1 (33, 34).

The DsbCn-DsbA, DsbCn-PDIa, and DsbCn-PDIb have also been predicted to have a similar V-shaped structure as DsbC, but with less reliability than that of DsbCn-Trx. Thus the packing between the two domains in DsbCn-DsbA, DsbCn-PDIa, and especially in DsbCn-PDIb, could be less compact so that the structure of the C-terminal part of DsbCn and the N-terminal part of the C-terminal domain could be disturbed by hybridization, even though the global three-dimensional structures of the two domains did not change much. In a loosely connected hybrid the linker helix would structurally be isolated. Thus, the structural relation of the two domains and the stability of the hybrid molecule would be further reduced. However, the hybrids of DsbCn-DsbA and DsbCn-PDIa are stable enough to be overexpressed as soluble dimeric molecules, exhibit similar CD spectra to that of DsbC, and have impressive isomerase activity and greatly elevated chaperone activity, which is only somewhat lower than that of DsbCn-Trx. The packing between the two domains in these hybrids should thus be compact enough to tolerate possible structural disturbance resulted from hybridization. Comparatively, the hybrid of DsbCn-PDIb shows a CD spectrum deviated in certain extent and significantly higher apparent molecular weight determined by exclusion chromatography possibly resulted from its structure being less compact. This is also consistent with the lower structure-prediction reliability and its rather low chaperone activity.

TABLE III
Enzymatic activities of domain hybrids

Protein	Protein-disulfide isomerase		Thiol-protein reductase ^a
	Specific activity ^a	Relative activity	
	unit/ μ mol	%	$\times 10^{-3} \Delta A_{650\text{nm}}/\text{min}^{-2}$
DsbC	14.6 \pm 1.4	100	2.49 \pm 0.19
PDI	62.3 \pm 14.8	426.7 \pm 101.4	
Trx	0	None, <4 ^b	3.05 \pm 0.20
DsbA	1.5 \pm 0.4	10.3 \pm 2.7	0.24 \pm 0.01
PDIa		None ^c	
DsbCn-Trx	4.8 \pm 0.8	32.9 \pm 5.5	0.76 \pm 0.05
DsbCn-DsbA	7.4 \pm 0.9	50.7 \pm 6.2	0.23 \pm 0.03
DsbCn-PDIa	24.4 \pm 0.6	167.1 \pm 4.1	0.10 \pm 0.01

^a Data for each monomer are expressed as mean \pm S.D. ($n = 3-5$).

^b Data calculated from Hawkins et al. (28).

^c Data from Ruoppolo et al. (29).

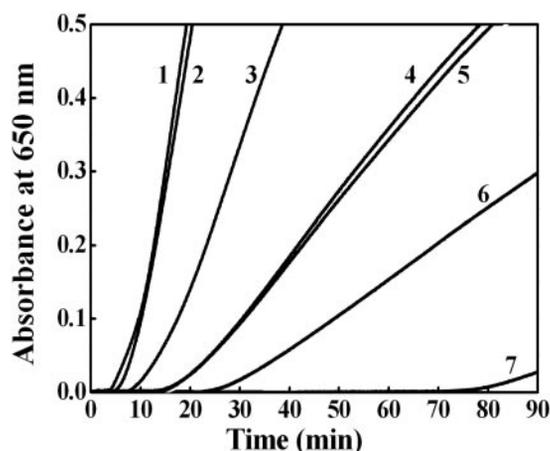


FIG. 4. Thiol protein reductase activity of domain hybrids. The assay was carried out by addition of 0.35 mM DTT to 0.1 M potassium phosphate, pH 6.6, containing 0.13 mM bovine insulin in the presence of 5 μ M protein, and the absorbance at 650 nm resulted from the aggregation of reduced insulin B chain was monitored immediately at 25 $^{\circ}$ C. Curve 1, Trx; curve 2, DsbC; curve 3, DsbCn-Trx; curve 4, DsbA; curve 5, DsbCn-DsbA; curve 6, DsbCn-PDIa; curve 7, DTT alone.

In prokaryotes the majority of disulfide bond-containing proteins are located in the periplasm and the outer membrane or secreted to the extracellular medium, and the formation of disulfide bond occurs in the oxidizing periplasm but not in the reducing cytoplasm. The bacterial periplasm is separated from the environment by only the permeable outer membrane and is therefore liable to be affected by environmental fluctuation. However, no classical molecular chaperone was identified in the periplasm for a long time (36). The Dsb family contains at least six members, which cooperate to stimulate the formation of correct disulfide bond(s). However, only few proteins in the periplasm, except alkaline phosphatase, contain multiple disulfide bonds (37). Therefore it is not clear why *E. coli* has two disulfide isomerases, DsbC and DsbG, which are not only homologous with 30% identity and 49% homology but also both dimeric molecules with Trx fold (38). We suggest that during evolution a thrifty and efficient way has evolved to make DsbC (and also possible DsbG) to function not only as an isomerase to catalyze the formation of correct disulfide bonds of multidisulfide-containing proteins (such as a number of virulence factors and toxins), but also as a chaperone by adding another domain to a Trx-fold molecule or a domain for dimerization in order to provide a space with hydrophobic surface for non-native folding intermediate binding. DsbC in prokaryotes may function as a chaperone more than an isomerase. At least in *in vitro* experiments DsbC showed only 20–30% of isomerase activity relative to that observed in the eukaryotic PDI, yet it exhibited more pronounced chaperone activity than PDI (5). Our data

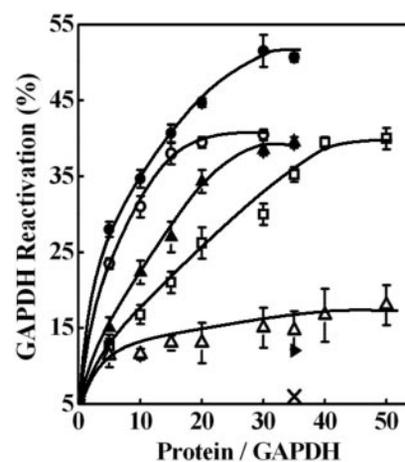


FIG. 5. Effect of the domain hybrids on the reactivation of GdnHCl-denatured GAPDH upon dilution. GAPDH at 140 μ M was denatured overnight in 3 M GdnHCl and 1 mM DTT at 4 $^{\circ}$ C. Reactivation was initiated by 50-fold dilution of the denatured GAPDH into 0.1 M phosphate buffer, pH 7.5, containing 2 mM EDTA and DsbC (\bullet), DsbCn-Trx (\circ), DsbCn-DsbA (\blacktriangle), DsbCn-PDIa (\square), DsbCn-PDIb (\triangle), DsbA (\blacktriangleright), or Trx (\times) at different molar ratios to GAPDH as indicated. The reactivation mixture was first kept at 4 $^{\circ}$ C for 1 h and then at 25 $^{\circ}$ C for another 2 h before an aliquot containing 0.2 μ g of GAPDH was taken for activity assay at 25 $^{\circ}$ C. The data were expressed as mean \pm S.D. ($n \geq 3$).

have strongly suggested that to link a Trx-fold molecule or a domain to the association domain of DsbC as a structural base to create a new dimeric molecule, and thus bestow new activities to the molecule to fulfill the requirement of the living organism, is very likely an efficient way to create new functions. In this regard, it has been suggested that DsbC evolved from Trx, and through the dimerization of Trx-domains of DsbC, its active site is protected by DsbB-mediated oxidation so that the oxidative and reductive pathways can co-exist in *E. coli* periplasm (10).

A recent report (39) has added one more experimental approach to support the rationale that modular organization of proteins is a widely used strategy for protein evolution. An evolutionary fusion of a Trx-like thiol-redox active module (the γ domain of DsbD) and a non-Trx-like (immunoglobulin-fold) thiol-redox active module (α domain of DsbD) resulted in the expansion of the substrate specificity of a pre-existing trans-membrane enzymatic core, CcdA or β domain of DsbD, bridging two seemingly unrelated thiol-redox pathways (39). The Trx fold has been used as a scaffold in many different proteins because it tolerates amino acid variability, additions, and insertions at a number of regions without affecting the overall fold (40). The recently published DsbE structure has provided one more example for how the commonly occurring Trx fold can



FIG. 6. **Predicted structure of DsbCn-Trx.** *A*, ribbon diagram of the superposition of DsbC monomer (in *black*) and DsbCn-Trx monomer (in *gray*) viewed normal to the molecular 2-fold axis, *i.e.* the linker helices (residues 62–77) in the center of the diagram. *B*, ribbon diagram of DsbCn-Trx homodimer. The monomers are shown in *black* and *gray*. The active site Cys residues are shown as *spheres*.

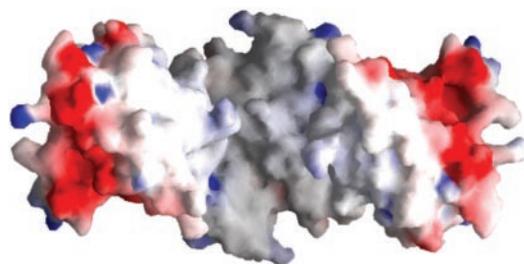


FIG. 7. **Surface representation of DsbCn-Trx viewed down the molecular 2-fold axis.** Electrostatic potential and molecular surface were calculated using GRASP (31). *Red* indicates negatively charged (-12 kT) and *blue* positively charged regions ($+13$ kT).

be modified quite simply to achieve a very specific function (41). DsbE, a membrane-anchored member of the Dsb family, has a modified Trx fold with an unusually acidic active site and an adjacent groove formed from two inserts in the fold. These two unique structural features have been suggested to be necessary for a specific reducing activity in an oxidizing periplasm and a high fidelity of interaction required for c-type cytochrome maturation. A ubiquitously expressed endoplasmic reticulum protein ERp 29 is composed of an N-terminal domain of P5-like PDIs and a C-terminal non-redox-active Trx-fold domain, which acts uniquely for dimerization, and may share the redox-independent chaperone activity of PDI using dimerization sites associated with the flexible linker region between the two domains as binding locus for unfolded polypeptides (42).

The report that isolated PDI *a* and *a'* domains show very

little isomerase activity has recently been confirmed (29), although they catalyze the oxidation of thiols. We have now shown that one DsbCn-PDIa subunit containing one active site exhibited 40% of isomerase activity of one PDI subunit containing two active sites, *i.e.* the *a* domain in the dimeric hybrid has retrieved almost all of its activity despite it being linked with the *b* domain in PDI molecule or with DsbCn in the hybrid DsbCn-PDIa molecule. Like DsbCn-PDIa, DsbCn-Trx and DsbCn-DsbA show a 5–10-fold increase in their isomerase activity relative to that observed in Trx and DsbA. To the best of our knowledge, the capacity to isomerize disulfide bonds has been reported only for multidomain proteins (29), such as Erp57, Erp72, and p5, besides PDI, DsbC, and DsbG discussed here, but not for monomeric Trx, DsbA, or isolated *a* and *a'* domain of PDI. However, the isomerase activity depends on the sequence -XY- in the active site -CXYC- and the redox potential of the active-site motif (43–45), therefore the activities of DsbCn-Trx and DsbCn-DsbA are only about 25% of the activity of DsbCn-PDIa.

The reductase activity of dimeric DsbCn-Trx is lower than that of monomeric Trx. This could be resulted from possible higher spatial accessibility and structural flexibility of the active site in the monomeric molecule.

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