Overproduction of Bacterial Protein Disulfide Isomerase (DsbC) and Its Modulator (DsbD) Markedly Enhances Periplasmic Production of Human Nerve Growth Factor in *Escherichia coli*

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Production of eukaryotic proteins with multiple disulfide bonds in the *Escherichia coli* periplasm often encounters difficulty in obtaining soluble products with native structure. Human nerve growth factor β (NGF) contains three disulfide bonds between nonconsecutive cysteine residues and forms insoluble aggregates when expressed in *E. coli*. We now report that overexpression of Dsb proteins known to catalyze formation and isomerization of disulfide bonds can substantially enhance periplasmic production of NGF. A set of pACYC184-based plasmids that permit *dsb* expression under the araB promoter were introduced into cells carrying a compatible plasmid that expresses NGF. The efficiency of periplasmic production of NGF fused to the OmpT signal peptide was strikingly improved by coexpression of DsbCD or DsbABCD proteins (up to 80% of total NGF produced). Coexpression of DsbAB was hardly effective, whereas that of DsbAC increased the total yield but not the periplasmic expression. These results suggest synergistic roles of DsbC and DsbD in disulfide isomerization that appear to become limiting upon NGF production. Furthermore, recombinant NGF produced with excess DsbCD (or DsbABCD) was biologically active judged by the neurite outgrowth assay using rat PC12 cells.

The periplasm of *Escherichia coli* contains enzymes that can assist protein folding such as Dsb (disulfide bond formation) proteins (1–6), like endoplasmic reticulum of eukaryotes, and can provide an oxidative environment potentially useful for production of heterologous secretory proteins (1–6), like endoplasmic reticulum of eukaryotes, and such structural features have been thought to create difficulties in obtaining active proteins, because incorrect disulfide bond formation is likely to yield inactive and insoluble products (13). Recent studies revealed several novel enzymes and factors involved in disulfide bond formation in the *E. coli* periplasm, which include at least four proteins, DsbA, DsbB, DsbC, and DsbD (4, 5, 14–17). These Dsb proteins contain one or more highly conserved thioredoxin-like Cys-Xaa-Xaa-Cys motifs that are crucial for the activity of disulfide oxidoreductases (5, 18). DsbA is a periplasmic enzyme that can act on nascent polypeptide chains in the formation of disulfide bonds during their folding (16, 19, 20). DsbC is another periplasmic enzyme known as a disulfide isomerase and can convert aberrant disulfide bonds to correct ones (16, 21–24). DsbB and DsbD are associated with the inner membrane and modulate activities of DsbA and DsbC, respectively (14, 15, 24, 25). Thus, an efficient chain of reactions for disulfide bond formation and isomerization seems to be operated in the periplasm during normal growth.

Based on the above findings, overexpression of Dsb proteins has been employed to increase efficiency of periplasmic expression of a number of heterologous proteins with multiple disulfide bonds in *E. coli* (4, 9, 10). So far, only limited success was reported; periplasmic expression of some proteins was improved by coexpression of DsbA, together with addition of reduced glutathione or *N*-acetylcysteine to the medium (26). DsbC can become overloaded upon production of heterologous proteins with multiple disulfide bonds, leading to insufficient conversion of aberrant disulfide bonds to the correct forms (4, 7, 27). A *dsbC* deletion reduced the production of urokinase (15) or insulin-like growth hormone I (28) that has disulfide bonds formed between nonconsecutive cysteine residues but hardly affected production of alkaline phosphatase or OmpA (15) or human growth hormone that has disulfide bonds between consecutive cysteine residues (28). These results indicated potentially important roles of DsbC in folding of proteins carrying multiple disulfide bonds, especially those involving nonconsecutive cysteine pairs. It should be noted that DsbC exhibits a disulfide reductase activity (the active site cysteine residues must be kept reduced to attack incorrectly formed disulfide bonds and catalyze isomerization (14, 15, 24)) in the highly oxidative periplasmic environment as compared with the endoplasmic reticulum of eukaryotes (2, 5, 13); the difference in the redox environment between the two compartments may be responsible for inefficient expression of some eukaryotic proteins in *E. coli* (13). The redox potential of the active site of DsbC is controlled by DsbD (14, 15, 24) in a fashion quite distinct from that of protein disulfide isomerase regulated by reduced glutathione in eukaryotes (5, 13, 29).

In this study, we systematically examined the effects of
Expression of NGF in E. coli Periplasm

Controlled coexpression of sets of Dsb proteins on periplasmic production of human nerve growth factor β (NGF). NGF carries three disulfide bonds with nonconsecutive cysteine pairs and was previously shown to aggregate upon periplasmic expression in E. coli (30). The set of newly constructed plasmids provided convenient means of assessing the effects of controlled coexpression of dsb genes on specific target proteins expressed from compatible plasmids. The results revealed a striking enhancement of periplasmic production of NGF, particularly when both DsbC catalyzing the isomerization and its membrane-associated modulator DsbD are simultaneously overexpressed.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli K12 strain JM109 (F’, recA1, endA1, gyrA96, thi-1, supE44, relA1, slac Δ proAB, (F’ traD36, proAB, lacIq lacZΔM15), mcrA) (Takara, Kyoto, Japan) was used as the expression host throughout the experiments. DM911 (dsbB null mutant of CA8000) (31) and SR2612 (dsbD null mutant of MC4100) (14) were kindly provided by D. Missiakas. Plasmid pT7Blue® was purchased from Novagen. Expression vector pAR2K for Dsb proteins was constructed by inserting a fragment of pTrc99A containing a rrnB7II2 terminator region into a derivative of pAR2, pACYC184-based arabinose promoter plasmid designed with ColEl-dependent DNA ligations (32). Expression vectors for the target protein NGF are derivatives of pTrc99A (Amersham Pharmacia Biotech), which allowed expression of NGF fused in frame to downstream of OmpA, OmpT, or MalE signal peptide, respectively, under controls of the trc promoter and the lacIq repressor and designated pTrc-OmpA, pTrc-OmpT, and pTrc-MalE, respectively. Vectors carrying each of the signal sequences (synthetic oligonucleotides) were constructed, and the coding region of the NGF gene (R & D Systems) was inserted downstream of pTrc-OmpA, pTrc-OmpT, and pTrc-MalE, and the resulting plasmids were designated pTrc-Omp-A NGF, pTrc-OmpT-NGF, and pTrc-MalE-NGF, respectively. Some codons rarely used in E. coli were replaced by those used more frequently to improve translational efficiency of the mature form of the NGF gene; TCA (Ser1 and Ser2), CCC (Pro5), and AGG (Arg9) were replaced by AGC, CCG, and CGC, respectively.

Construction of Dsb Expression Plasmids—Each dsb gene (plus the respective or slightly modified Shine-Dalgalno sequence) was cloned by polymerase chain reaction (PCR) as follows: dsbA, dsbB, and dsbC genes were amplified with KOD DNA polymerase (Toyobo, Osaka, Japan), and dsbD was amplified with LA Taq DNA polymerase (Takara, Osaka, Japan). dsbA was amplified using

5′-CCGGAGTCATCGGAGAGTAGATGAGACGC-3′

and

5′-CGGGCCGCGGCGCGCGCTGAGATCACTGTA-3′

using 5′ GGCGAGCTCATCGGAGAGTAGATGAGACGC 3′ and primers pSki220 (33) as template, and the PCR product was digested with SacI and Aval, purified, and cloned into the SacI-AvaI site of pT7Blue®. Similarly, dsbB was amplified using 5′ GGCGAGCTCATCGGAGAGTAGATGAGACGC 3′ and primers pSK220 (33) as template, and the PCR product was digested with Aval and NdeI, purified, and cloned into the Aval-NdeI site of pT7Blue®. dsbA was amplified using 5′ GGCGAGCTCATCGGAGAGTAGATGAGACGC 3′ and primers pSSSI (34) as template, and the PCR product was digested with Aval and NdeI, purified, and cloned into the Aval-NdeI site of pT7Blue®. dsbA was amplified using 5′ GGCGAGCTCATCGGAGAGTAGATGAGACGC 3′ and primers pSSSI (34) as template, and the PCR product was digested with Aval and NdeI, purified, and cloned into the Aval-NdeI site of pT7Blue®. dsbB was amplified using 3′ GGCGAGCTCATCGGAGAGTAGATGAGACGC 3′ and primers pSSSI (34) as template, and the PCR product was digested with Aval and NdeI, purified, and cloned into the Aval-NdeI site of pT7Blue®. The structure of the resulting artificial set of the dsb genes for four combinations was constructed using the SacI-AvaI fragment (~0.6 kb) of pTdsbA and the SacI-AvaI site of pTdsbB or pTdsbC to yield pTdsbAB or pTdsbCD, respectively. For construction of the entire set of dsb genes, the SacI-NdeI fragment (~1.1 kb) of pTdsbAB was inserted into the SacI-NdeI site of pTdsbCD to yield pTdsbABCD.

Each subset of the genes obtained above was placed under the control of the araB promoter and the araC regulatory gene on a pACYC184-based vector (pAR2K); the SacI-HindIII fragment of pTdsbAB, pTdsbBC or pTdsbCD, respectively, was inserted into the SacI-HindIII site of pAR2K, yielding pDbaB1, pDbaC1, pDbaB1, pDbcD1, or pDabcD1, respectively. pDabcD1 is a kanamycin-resistant version of pDabcD1 that was constructed by inserting the BstBI-BstII fragment of pDabcD1 (~4.8 kb) into pAR3kan carrying kanamycin (instead of chloramphenicol) resistance gene.

Culture Conditions and Protein Expression—E. coli JM109 cells carrying NGF such as pT7Blue®-NGF and a pDsb plasmid were grown in L broth (350 ml) supplemented with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) at 37 °C. When a culture reached 20 Klett units (number 66 filter), Dsb proteins were induced by adding l-arabinose to the medium, and 30 min later, NGF was induced by adding 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG). All chemicals were of analytical grade supplied by Wako Pure Chemical (Osaka, Japan) or Sigma (St. Louis, MO).

Fractionation of Proteins—Samples of cells (200 μl) were harvested by centrifugation, and periplasmic proteins were obtained by osmotic disruption of spheroplasts; cells were first resuspended into 100 μl of 30 mM Tris-HCl (pH 8.0), 20% sucrose, lysozyme was added to 0.1 mg/ml, and cells were incubated at 4 °C for 30 min. After adding MgCl2 to 50 mM, the sample was centrifuged at 20,000 × g for 10 min, and the supernatant was taken as the fractionated cell extract and was resuspended in 100 μl of 5 mM MgCl2, sonicated for 10 min, centrifuged at 100,000 × g at 4 °C for 1 h on a Beckman TL-A120 rotor, and the supernatant was withdrawn as the cytoplasmic fraction. The resulting pellet was resuspended in 100 μl of 5 mM MgCl2, 1% octylglucoside, incubated at 4 °C for 10 min, and centrifuged again at 100,000 × g at 4 °C for 1 h. The supernatant was used as the membrane fraction, and the pellet was used as the insoluble fraction. Whole-cell proteins were prepared separately by precipitating a portion (200 μl) of the culture directly with trichloroacetic acid (36).

Analysis of Proteins—Each of the above fractions was resuspended in SDS sample buffer, either directly or after trichloroacetic acid precipitation and washing with acetone, and heat-treated essentially as described (37). Proteins (corresponding to equal optical density) were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) followed by protein staining with Coomassie Brilliant Blue or by immunoblotting with specific polyclonal antibody against NGF (Santa Cruz Biotechnology, Inc.). DsbA (kindly donated by Y. Akiyama), β-lactamase (5 Prime → 3 Prime, Inc., Boulder, CO), alkaline phosphatase (Nordic Immunological Laboratories, Inc.), and β-galactosidase (StressGen, Inc.). The detection system used was either horseradish peroxidase-conjugated anti-rabbit or -mouse antibody and an ECL kit (Amersham Pharmacia Biotech) or horseradish peroxidase-conjugated anti-rabbit or -mouse antibody and 3,3′,5,5′-tetramethylbenzidine (Bio-Rad, Inc.). Quantification was carried out by a Densi-Scan apparatus (BioImage Systems Co., Tokyo, Japan).

Bioassay for NGF Activity—E. coli JM109 cells carrying pT7:Omp-NGF and pDbCD1 (or pDabcD1 or vector alone) was grown in L broth (400 ml × 2) and induced for expression of Dsb proteins followed by that of Omp2 (30) for 8 h. Cells were harvested and washed with 0.85% NaCl, and 4 g of wet cells were resuspended in 40 ml of buffer containing 0.1% (v/v) protease inhibitor mixture (Sigma). Periplasmic fraction was prepared and concentrated by a Centriprep 10 concentrator (Amicon) and the buffer was adjusted to 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and adsorbed to Affi-prep Polymixin Matrix (Bio-Rad) by shaking overnight on a rotary shaker at 4 °C. After centrifugation at 10,000 g for 10 min, the supernatant was dialyzed against 20 mM Tris-HCl (pH 8.0), 1 mM EDTA and applied to a DEAE Toyopearl column (Tosoh, Tokyo, Japan). After washing the column with the same buffer containing 0.2 mM KCl, NGF was eluted with the buffer containing 0.5 mM KCl. The eluate was concentrated on a Centricon 10 concentrator (Amicon) to ~2 ml and dialyzed against 20 mM Tris-HCl (pH 8.0), 1 mM EDTA. This procedure removed >80% of proteins without apparent loss of NGF activity.

The partially purified NGF thus obtained was assayed for activity by adding serial dilutions of sample to rat pheochromocytoma (PC12) cells (30) grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum and 10% horse serum using 24-well collagen-coated plates. Mouse NGF (Biomedical Technologies Inc.) served as a standard for comparison. Neurite outgrowth was observed under a microscope after incubation of plates at 37 °C in 5% humidified CO2 for 7 days.

The abbreviations used are: NGF, nerve growth factor; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair.

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RESULTS

Construction of the dsb Expression Plasmids—The dsbA, dsbB, dsbC, and dsbD genes were cloned and tandemly joined in various combinations to obtain artificial operons under the araB promoter on a pACYC184-based plasmid, pARK2. Each of the dsb genes contained the entire coding region with short flanking regions on both sides including a putative Shine-Dalgarno sequence but not a transcription terminator. Several pairwise combined genes (dsbAB, dsbAC, dsbCD, and dsbBD) and the complete set of genes (dsbABCD) were initially cloned on a high copy plasmid as described under “Experimental Procedures.” Each set of the genes was then placed under the araB promoter controlled by the araC regulatory gene on pARK2 vector, and the resulting plasmids were designated pDbAB1, pDbAC1, pDbBD1, pDbCD1, and pDbABCD1 (Fig. 1). These plasmids are compatible with ColE1-type plasmids generally used for expression of recombinant proteins.

When l-arabinose was added to an L broth culture of E. coli carrying each of the expression plasmids, the respective Dsb proteins were induced to various levels depending on the arabinose concentration used (Fig. 2B). DsbA protein (21 kDa) was detected by Western blotting with DsbA-specific antibody, whereas DsbC protein (24 kDa) was detected by staining with Coomassie Brilliant Blue on SDS-PAGE (Fig. 2A), although the mobility observed was appreciably slower than that predicted from the primary structure as observed previously by Missiakas et al. (21). On the other hand, expression of DsbB and DsbD from the plasmids was confirmed by their abilities to complement the defective phenotypes (higher sensitivity to dithiothreitol or to CuSO₄) of the respective deletion mutants (data not shown). The growth of host bacteria carrying pDbABCD1 that can express all the Dsb proteins was not affected significantly by addition of l-arabinose up to 200 µg/ml, which was adopted as the standard condition.

Inhibition of Cell Growth by NGF and Its Relief by Dsb Coexpression—The human NGF gene was fused with a signal peptide of ompA, ompT, or malE to facilitate membrane transport and was expressed in JM109 cells. When these NGF fusion proteins were induced by adding 0.1 mM IPTG at 37 °C, their production was detected within 30 min, gradually increased for about 90 min, and cell growth was retarded depending on the kind of signal peptide used. When the ompA or malE signal was used, a marked growth inhibition was observed, whereas the ompT signal caused only slight inhibition (Fig. 3A). Such differential effects of the various signal peptides suggested that certain anomaly in membrane transport of NGF fusion protein caused inhibition of cell growth. Indeed, the amount of periplasmic enzyme β-lactamase was clearly reduced upon induction of OmpA-NGF or MalE-NGF (data not shown).

We then introduced the Dsb expression plasmid (pDbABCD1) into the above strains and examined the effects of Dsb coexpression on cell growth. The growth inhibition observed upon OmpA-NGF or MalE-NGF expression was found to be exacerbated when Dsb proteins were coexpressed; no clones carrying both the OmpA-NGF and pDbABCD1 (or pDbAB1) expression plasmids were obtained even after prolonged incubation. Although a clone carrying both OmpA-NGF and pDbCD1 expression plasmids was obtained, no accumulation of NGF or β-lactamase was observed (data not shown). In contrast, the slight but significant growth inhibition observed upon OmpT-NGF expression was completely relieved when the Dsb proteins were coexpressed by addition of l-arabinose (Fig. 3B). These results suggested that OmpT-NGF fusion protein can be successfully transported to the periplasm at least to some extent and that membrane transport of this, as well as other periplasmic proteins, is enhanced by overproduction of Dsb proteins in the periplasm.

Effects of Dsb Coexpression on the Production and Localization of NGF—To determine whether overexpression of the Dsb proteins enhances production of NGF possibly through assisting the folding of OmpT-NGF, various sets of Dsb proteins were coexpressed from the expression plasmids, and their effects on the amount of OmpT-NGF produced and on periplasmic localization were compared. Coexpression of DsbAB or DsbCD should enhance the efficiencies of either disulfide bond formation or isomerization, respectively, whereas that of DsbAC increases both the disulfide bond formation and isomerization activities. Under these conditions, coexpression of OmpT-NGF did not affect the levels of DsaA and DsaC significantly (data not shown).

As shown in Fig. 4, the control cells carrying the pACYC184 vector produced NGF of which about 30% was found in the periplasmic fraction after induction for 3.5 h (lanes 1 and 6). Coexpression of DsbAB hardly affected the total or periplasmic expression of NGF (lanes 2 and 7), whereas that of DsbAC increased the total yield nearly 2-fold but hardly increased the periplasmic production (lanes 3 and 8). Essentially the same result was obtained when the level of DsbAC coexpression was further enhanced by using a higher concentration of arabinose (data not shown). In contrast, coexpression of DsbCD enhanced the total NGF production by about 2-fold and the periplasmic expression by about 3-fold over the vector control; namely, about 60% of total NGF was recovered in the periplasm (lanes 4 and 9). Even higher periplasmic production of NGF (~80%) was observed when all the Dsb proteins (DsbABCD) were overexpressed (lanes 5 and 10). In the presence of excess DsbCD or DsbABC (but not DsbAB), NGF was significantly stabilized, which probably explains the increase in total NGF production (data not shown).

Effects of Varying Levels of DsbABCD Coexpression on Localization of NGF—To further assess the effects of DsbABCD coexpression on OmpT-NGF production, we varied the level of Dsb coexpression and analyzed its effect on distribution of NGF into several distinct subcellular fractions. The extent of Dsb
coexpression, within the range tested, hardly affected the total amount of NGF produced, whereas NGF obtained in the periplasmic fraction increased markedly with increasing level of Dsb coexpression (Fig. 5A), concomitant with the decrease in the insoluble fraction (Fig. 5B). Again, about 80% of the total NGF produced was found in the periplasm at the maximum Dsb coexpression. Only the band with mobility characteristic of the mature NGF was detected, suggesting that the signal peptide was effectively removed from the precursor OmpT-NGF by processing. The periplasmic fraction analyzed contained most of β-lactamase and alkaline phosphatase as expected but very little cytoplasmic protein, DnaJ (Fig. 5A). Thus, NGF found in the periplasmic fraction most probably represents soluble forms of protein that had been successfully processed and correctly folded in the periplasm.

**Periplasmic Production of Active NGF by Dsb Coexpression**—To further substantiate the above possibility, we determined the biological activity of soluble NGF found in the periplasm by using neurite outgrowth assay with rat PC12 cells. To remove proteins toxic to the cells while minimizing possible denaturation or refolding of NGF produced, the periplasmic fraction was concentrated without salt precipitation followed by treatments with affinity and ion-exchange columns. Bioassays with serial dilutions of product revealed that NGF found in the periplasm upon DsbCD (or DsbABCD) overexpression exhibit activity comparable with that of authentic mouse NGF (Fig. 6, B and C). On the other hand, the NGF recovered from similar periplasmic fraction obtained without overexpression of Dsb proteins, even after 4-fold concentration, failed to show any detectable activity (Fig. 6A), indicating that NGF produced under these conditions is hardly active. These results strongly suggest that NGF produced and transported to the periplasm in *E. coli* become biologically active only when assisted by extensive overexpression of DsbCD (or DsbABCD).

**Fig. 2.** Expression of Dsb proteins from the expression plasmids. Strain JM109 carrying each of the Dsb expression plasmids or the vector (pARK2) was grown in L broth containing chloramphenicol (34 µg/ml) at 37 °C, and Dsb proteins were induced with L-arabinose for 1 h. Expression of Dsb proteins was analyzed by SDS-PAGE using a 12.5% acrylamide gel followed by staining with Coomassie Brilliant Blue (upper panels) or by immunoblotting of the same gel using DsbA-specific antibody (lower panels). Numbers to the left indicate molecular mass (kDa) of protein markers (Bio-Rad). A, expression of DsbA and DsbC proteins with 200 µg/ml of L-arabinose. The asterisk (*) represents a nonspecific band and not DsbC. B, dependence of Dsb expression on L-arabinose concentration.

**Fig. 3.** Effects of NGF production and overexpression of Dsb proteins on cell growth. A, effects of production of NGF fused with diverse signal peptides. Derivatives of strain JM109 carrying pTrc-OmpA-NGF ( ), pTrc-OmpT-NGF ( ), pTrc-MalE-NGF ( ), or pTrc-OmpT vector ( ) were grown in L broth containing ampicillin at 37 °C. When the culture reached 20 Klett units, expression of NGF was induced by adding 50 µM IPTG. B, effects of coexpressing OmpT-NGF and DsbABCD. JM109 cells carrying both pTrc-OmpT-NGF and pDsbABCD ( ) or pTrc-OmpT-NGF and pACYC184 vector ( ) were grown in L broth containing ampicillin and chloramphenicol at 37 °C, and when the culture reached 20 Klett units, expression of Dsb proteins was induced by L-arabinose (L-ara; 200 µg/ml), followed by induction of OmpT-NGF with 50 µM IPTG after 30 min.
DISCUSSION

We have constructed a set of versatile plasmids for controlled expression of Dsb proteins to assess the effects of coexpression on periplasmic production of recombinant proteins in E. coli. These plasmids permit coordinate induction of different sets of Dsb proteins by manipulating their expression levels and timing independently from that of the target recombinant protein. The maximum extents of overproduction for DsbA and DsbC proteins were severalfold higher than the normal levels but hardly affected cell growth under the conditions employed.

Among the three signal peptides tested, the OmpT signal turned out to be most effective for producing soluble NGF with apparently little effects on translocation of periplasmic proteins and host cell growth, and the slight inhibitory effect on growth was overcome by overexpressing the whole set of Dsb proteins (Fig. 3). In contrast, when the OmpA or MalE signal was used, marked growth inhibition was observed upon induction of NGF presumably because of defective membrane translocation of secretory proteins; however, this defect was not rescued by overproduction of Dsb proteins. Although the mechanism of secretion defects remains obscure, the N terminus of mature NGF containing three consecutive serine residues tends to form a β-turn structure and could be involved in translocation inhibition of NGF precursor. The present results revealed that such inhibition can be alleviated by using an appropriate signal peptide.

Among the subsets of Dsb proteins tested, coexpression of DsbCD but not DsbAB was effective and that of DsbABCD was most effective for obtaining soluble NGF in the periplasm (Fig. 4). Thus, although coexpression of DsbAB alone has little effect, that of both DsbAB and DsbCD are highly effective perhaps through synergistic function between two pairs of proteins in assisting folding of NGF. Although DsbCD could assist translocation of the target protein directly or indirectly by pulling the precursor into the periplasm (17), it seems more likely that overexpressed DsbCD proteins efficiently promoted isomerization of aberrant disulfide bonds formed on nascent NGF, thus yielding correctly folded products. Sone et al. (16) demonstrated that overexpressed DebC acts as disulfide isomerase on the mutant alkaline phosphatase, which contains aberrant disulfide bonds formed between consecutive cysteine residues. However, only a limited success was reported on disulfide isomerization of mature NGF with the OmpT signal, suggesting that the OmpT signal is not suitable for expressing NGF in E. coli. Therefore, we further tested the OmpT signal for producing soluble NGF in combination with other signals.

FIG. 4. Effects of coexpression of different subsets of Dsb proteins on OmpT-NGF production. Derivatives of strain JM109 carrying both pTrc-OmpT-NGF and a Dsb expression plasmid (or pACYC184 vector) were grown in L broth, and expression of Dsb proteins and OmpT-NGF was induced as described in the legend to Fig. 3. After induction of OmpT-NGF for 3.5 h, whole-cell proteins (lanes 1–5) and periplasmic proteins (lanes 6–10) were prepared separately from equal volumes of each culture. Proteins were analyzed by SDS-PAGE (15% gel) followed by immunoblotting for NGF and β-lactamase (Bla) and quantified as described under “Experimental Procedures.” Values shown below the blots indicate amounts of NGF obtained relative to that found in whole-cell proteins from the vector control (lane 1).

FIG. 5. Effects of varying levels of DsbABCD coexpression on intracellular distribution of OmpT-NGF. Strain JM109 carrying both pTrc-OmpT-NGF and pDbABCD1 was grown, and Dsb proteins and OmpT-NGF were induced as described in the legend to Fig. 3. After a 1-h induction of OmpT-NGF, cells were collected, fractionated, and analyzed as described under “Experimental Procedures.” A, effects of Dsb coexpression level on the periplasmic expression of OmpT-NGF. Whole-cell proteins (lanes 1–4) and periplasmic proteins (lanes 5–8) were prepared from equal volumes of cultures, analyzed by SDS-PAGE (15% gel) followed by immunoblotting with a mixture of antibodies against NGF, DnaJ, β-lactamase (Bla), alkaline phosphatase (PhoA) and PhoA-conjugated secondary antibody. B, effects of Dsb coexpression level on the intracellular localization of OmpT-NGF. Whole-cell proteins (W), periplasm (P), cytoplasm (C), membrane (M), and insoluble fractions (I) were prepared from equal volumes of cultures, analyzed by SDS-PAGE, immunoblotted as in A, except that the secondary antibody used was horseradish peroxidase-conjugated antibody, and quantified as described under “Experimental Procedures.” Cytoplasmic fraction is not shown, because the amount of NGF detected was negligible for all cultures examined.
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In the course of the present study, Qiu et al. (38) reported production of a soluble and active human tissue plasminogen activator that has 527 amino acid residues and 17 nonconsecutive disulfide bonds in the E. coli periplasm overexpressing DsbC (38). However, in this case, overexpressed DsbC might act stoichiometrically rather than catalytically, and the inactive DsbC could inhibit the membrane transport of precursor DsbC and other proteins essential for cell growth. The present results based on the differences between DsbAC and DsbCD coexpression suggested that overexpression of DsbC is necessary but not sufficient for obtaining maximum periplasmic production of NGF; coexpression of DsbC with DsbD, which is presumably required for regenerating DsbC, appeared to be crucial for efficient isomerization of disulfide bonds in the E. coli periplasm. Consistent with this proposal, expression of a bovine pancreatic trypsin inhibitor containing three disulfide bonds (nonconsecutive-type) yielded intermediates mostly with aberrant disulfide bonds in a dsbD deletion mutant (14). Similarly, the yield of active human plasminogen activator containing three disulfide bonds (nonconsecutive-type) was low, whereas bacterial alkaline phosphatase was normally produced in the dipZ (dsbD) mutant presumably because of formation of aberrant disulfide bonds (39). It thus seems clear that DsbD, as well as DsbC, can become overloaded upon overexpression of heterologous proteins having multiple disulfide bonds.

Because the increasing levels of DsbABCD coexpression enhance the amount of soluble NGF found in the periplasm with concomitant decrease in insoluble products, the overproduced Dsb proteins are most likely to enhance the periplasmic folding of NGF by preventing aggregation. Coexpression of DsbAB was hardly effective in this respect, suggesting that enhanced disulfide-forming activity alone is not sufficient for facilitating the protein folding. In contrast, coexpression of DsbAC, DsbCD, or DsbABCD markedly enhanced the total amount of NGF produced (Fig. 4). Overexpression of DsbC therefore appears to protect the product in some way from proteolysis; in fact, the NGF product was stabilized in the presence of excess DsbCD or DsbABCD proteins. On the other hand, increased periplasmic production of NGF was observed only when DsbC and DsbD were simultaneously coexpressed (DsbCD or DsbABCD). This suggests that DsbC can assist conversion of aberrant disulfides of NGF to the native form but cannot release native, soluble products in the absence of sufficient amounts of DsbD. The results of bioassay revealed that recombinant NGF produced with DsbCD overexpression has activity similar to that of authentic NGF, suggesting that correct folding of NGF was efficiently catalyzed by overexpressed DsbCD proteins. Detailed mechanisms of disulfide isomerization including the mode of modulation of DsbC activity by DsbD or other factors should have important bearings on further understanding and improving of periplasmic production of recombinant proteins that require complex disulfide bond formation.

After completion of this work, two laboratories reported correction of the primary structure of DsbD (40, 41), which indicated that translation of the dsbD gene begins at a start codon 76 codons upstream of that previously thought. Because the present expression plasmids were constructed on the basis of previously reported gene structure, the excess DsbD proteins obtained here lack the N-terminal 76 amino acid residues (including 26 possible signal sequences). However, the dsbD gene used did complement the defective phenotype of dsbD null mutant, and overexpression of DsbCD (but not DsbAC) exerted distinct effects on the periplasmic expression of NGF (Fig. 4), suggesting active participation of the excess DsbD produced; the truncated DsbD should retain all the cysteine residues that may be required for the disulfide oxidoreductase activity. We therefore believe that the essential finding and conclusion of this work remains unaffected, although the effect of DsbD overexpression observed here may have been underestimated.

In conclusion, our results strongly suggest that disulfide bond isomerization of NGF can be efficiently and synergistically catalyzed by overexpression of DsbC and its modulator.
DbD. Excess DbC protein appears to be successfully transported to the periplasm, and its activity can be effectively maintained by simultaneous supply of excess DbD. We also found recently that periplasmic production of horseradish peroxidase containing multiple disulfide bonds and unstable in *E. coli* is much improved by overexpressing Db proteins (42). The Db coexpression plasmids such as those reported here should prove useful for studying production of heterologous proteins with multiple disulfide bonds and prone to aggregation or degradation upon secretion to the periplasm.

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