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Respiratory chain strongly oxidizes the CXXC motif of DsbB in the *Escherichia coli* disulfide bond formation pathway

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Escherichia coli **DsbB has four essential cysteine residues, among which Cys41 and Cys44 form a CXXC redox active site motif and the Cys104–Cys130 disulfide bond oxidizes the active site cysteines of DsbA, the disulfide bond formation factor in the periplasm. Functional respiratory chain is required for the cell to keep DsbA oxidized. In this study, we characterized the roles of essential cysteines of DsbB in the coupling with the respiratory chain. Cys104 was found to form the inactive complex with DsbA under respiration-defective conditions. While DsbB, under normal aerobic conditions, is in the oxidized state, having two intramolecular disulfide bonds, oxidation of Cys104 and Cys130 requires the presence of Cys41–Cys44. Remarkably, the Cys41–Cys44 disulfide bond is refractory to reduction by a high concentration of dithiothreitol, unless the membrane is solubilized with a detergent. This reductant resistance requires both the respiratory function and oxygen, since Cys41–Cys44 became sensitive to the reducing agent when membrane was prepared from quinone- or heme-depleted cells or when a membrane sample was deaerated. Thus, the Cys41–Val–Leu–Cys44 motif of DsbB is kept both strongly oxidized and strongly oxidizing when DsbB is integrated into the membrane with the normal set of respiratory components.**

Keywords: CXXC motif/disulfide bond/DsbB/
Escherichia coli/respiratory chain

Introduction

Disulfide bonds are introduced into a number of proteins located outside the cytosol. They contribute to the stability of these proteins, and they are sometimes essential for the protein's folding into the native structure (Derman and Beckwith, 1991; Akiyama and Ito, 1993). Recent studies established that the formation of protein disulfide bonds in the periplasmic space of *Escherichia coli* cells is facilitated by multiple Dsb factors (for a review, see Missiakas and Raina, 1997). Yeast cells may also possess similar mechanisms in the endoplasmic reticulum (Frand and Kaiser, 1998; Pollard *et al*., 1998). The *E.coli* DsbA directly introduces disulfide bonds into newly synthesized proteins upon their translocation across the plasma membrane (Bardwell *et al*., 1991; Akiyama *et al*., 1992; Kamitani *et al*., 1992; Zapun and Creighton, 1994). DsbC isomerizes disulfide bonds until they are established between the correct combinations of cysteine residues (Zapun *et al*., 1995; Rietsch *et al*., 1996; Sone *et al*., 1997).

These periplasmic Dsb proteins have a characteristic Cys–X–X–Cys sequence motif (CXXC motif) found in a number of redox-active proteins such as thioredoxin (for a review see Chivers *et al*., 1997). It is remarkable that the redox states of the thioredoxin-like motifs in DsbA and DsbC are regulated by specific membrane proteins. Thus, DsbD maintains the CXXC motif in DsbC in the reduced states (Rietsch *et al*., 1996, 1997; Joly and Swartz, 1997). This reducing power seems to come from NADH through thioredoxin in the cytosol (Rietsch *et al*., 1996, 1997). In contrast, DsbB maintains DsbA in the oxidized state (Bardwell *et al*., 1993; Missiakas *et al*., 1993). The Cys30 residue of DsbA is unusually low in pK_a and very reactive, making the reduced form of this protein energetically favored (Zapun *et al*., 1993; Nelson and Creighton, 1994; Darby and Creighton, 1995; Grauschopf *et al*., 1995). Nevertheless, DsbA is kept completely oxidized in normal cells (Kishigami *et al*., 1995a), enabling the Cys30–Cys33 disulfide bond to oxidize directly a pair of cysteines on target proteins in the periplasm. As a result, Cys30 and Cys33 are reduced but they are reoxidized immediately by DsbB. This membrane protein has two pairs of essential cysteines, Cys41 and Cys44 in the N-terminally located periplasmic loop and Cys104 and Cys130 in the C-terminal periplasmic domain (Jander *et al*., 1994). Evidence indicates that a disulfide bond formed between the latter cysteine pair acts directly to oxidize the Cys30 and Cys33 residues of DsbA (Guilhot *et al*., 1995; Kishigami *et al*., 1995b; Kishigami and Ito, 1996). Although the amino acids, Val and Leu, flanked by Cys41 and Cys44 are not typical of those found in the corresponding positions of various redox-active proteins (Grauschopf *et al*., 1995; Chivers *et al*., 1997), the Cys41–Val–Leu–Cys sequence may be regarded as a thioredoxin-like CXXC motif in DsbB. We previously proposed that this CXXC motif reoxidizes the Cys104 and Cys130 residues in the same protein (Kishigami and Ito, 1997). Given this model, a question still remains about how Cys41 and Cys44 are reoxidized.

We showed previously (Kobayashi *et al*., 1997) that *E.coli hemA* and *ubiA menA* mutants, defective in respiration, accumulated a reduced form of DsbA when the mutant cells were growing under protoheme- or quinone-deprived conditions. Under such conditions, DsbB was converted into a form of DsbA–DsbB complex in which these two proteins were disulfide-linked, followed by reduction of the remaining molecules of DsbA in the cell. Eventually, accumulation of reduced β-lactamase became evident. These results suggest that the functional respiratory chain is required for DsbB to complete its reaction of oxidizing DsbA. Thus, in aerobically growing *E.coli* cells, the oxidizing equivalent for the formation of protein

disulfide bonds appears to be provided by oxygen through the respiratory chain, which seems to activate DsbB.

In the present study, we characterized redox states of the essential cysteine residues of DsbB, and found a peculiar property of its Cys41–Val–Leu–Cys44 CXXC motif. In intact cells or in isolated membranes, these cysteine residues are always kept oxidized and are even refractory to reduction by a reducing agent. This resistance against reduction requires that DsbB is integrated into the membrane with the normal set of respiratory components.

Results

In vivo redox states of the essential cysteine residues of DsbB

In the previous study (Kishigami and Ito, 1996), we showed that DsbB at steady state contains at least one disulfide bond, between Cys104 and Cys130, that is donated to DsbA. We noted subsequently that the 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) alkylation method (Vestweber and Schatz, 1988; Uchida *et al*., 1995) is useful for determining redox states of cysteine-containing proteins (Kobayashi *et al*., 1997). Since AMS modification of a free SH group increases the molecular mass by ~540 Da, this method was expected to enable simple SDS–PAGE to resolve protein isoforms with different numbers of reduced cysteines. We introduced Cys/Ser substitution mutations into the plasmid-cloned *dsbB-his$_6$-myc* gene, encoding DsbB with a C-terminally attached His$_6$-Myc epitope tag with full biological activity (Kishigami and Ito, 1996). The mutations were targeted to the four essential cysteines, Cys41, Cys44, Cys104 and Cys130 (Jander *et al*., 1994). The resulting mutant proteins are named according to the four character notations in brackets (e.g. DsbB[CCSS]), in which C and S indicate whether the residues 41, 44, 104 and 130 (in this order) are Cys or Ser. The mutant proteins were expressed in a strain deleted for the chromosomal *dsbB* gene. To avoid artificial thiol–disulfide exchange reactions after cell disruption (Kishigami *et al*., 1995a), we intended, whenever possible, to treat a bacterial culture directly with trichloroacetic acid (TCA). Protein precipitates were then dissolved in buffered SDS solution containing AMS, separated by SDS–PAGE without any reducing agent, and DsbB species were detected by Western blotting using anti-Myc antibodies.

We first established that DsbB isoforms or variants having different numbers of cysteines are electrophoretically separable. To reduce the cysteine residues fully, the TCA-denatured proteins were treated with dithiothreitol (DTT) as described in Materials and methods, and then modified with AMS in SDS solution. DsbB[CCCC], DsbB[SCCC], DsbB[SSCC]/DsbB[CCSS], DsbB[CSSS] and non-reduced DsbB[CCCC] exhibited distinct and increasing mobilities in this order (Figure 1, lanes 2–7), consistent with modification of n, $n-1$, $n-2$, $n-3$ and $n-4$ residues ($n = 6$, if non-essential cysteines are counted; see below), respectively. Other mutant proteins exhibited consistent electrophoretic mobilities (data not shown), although even proteins with the same number of cysteines sometimes differed slightly in their mobilities (for instance, note the mobility difference between DsbB[SSCC] and DsbB[CCSS] in Figure 1). Although

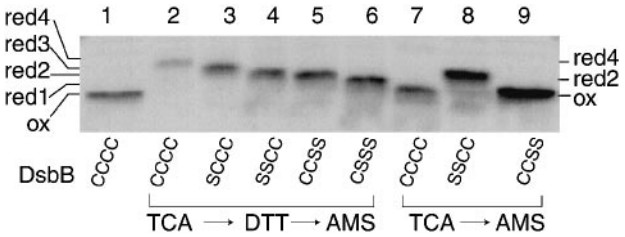

Fig. 1. Electrophoretic separation of DsbB and its Cys/Ser mutants after AMS modification. Strain TA164 (*dsbB*::*kan*) was transformed with one of the following plasmids expressing DsbB-His$_6$-Myc or its derivatives with Cys/Ser substitution(s) at residues 41, 44, 104 or 130; pSS51 for DsbB[CCCC] (lanes 1, 2 and 7), pSS53 for DsbB[SCCC] (lane 3), pTAK8 for DsbB[SSCC] (lanes 4 and 8), pTAK10 for DsbB[CCSS] (lanes 5 and 9) and pTAK18 for DsbB[CSSS] (lane 6). Cells were grown at 37°C in L-glucose medium, and portions of exponentially growing cultures were treated directly with TCA (final 5%) to precipitate whole-cell proteins. The protein precipitates were washed with acetone and either directly dissolved in SDS-Tris–HCl-AMS solution (lanes 1, 7, 8 and 9) or reduced with 100 mM DTT before the second TCA precipitation and solubilization in SDS-Tris–HCl-AMS (see Materials and methods). Proteins were separated by SDS–PAGE (without any reducing agent), and visualized with Western blotting using anti-Myc antibodies. ox indicates the position of the non-reduced DsbB, whereas red1, red2, red3 and red4 indicate the positions of DsbB with 1–4 additional modifications at reduced cysteines.

DsbB contains additional two non-essential cysteines, one in the cytoplasmic part and the other at the periplasmic end of a transmembrane segment (Jander *et al*., 1994), the topological separation precludes their disulfide bond formation. In fact, they seem to be always reduced and AMS-modifiable (Kobayashi *et al*., 1997), but we disregard these background modifications. Thus, only the modifications that occurred on the essential cysteine residues are counted; for instance, red2 in Figure 1 indicates DsbB in which two out of the four cysteines, at residues 41, 44, 104 and 130, have been modified.

From the results obtained, it is clear that four reduced cysteines were generated upon reduction of the denatured DsbB[CCCC] molecules with DTT (see below for the importance of denaturation). Therefore, it is concluded that DsbB normally contains two disulfide bonds formed among the essential four cysteines. Since the Cys104–Cys130 disulfide bond was already suggested to exist (Kishigami and Ito, 1996), we conclude that Cys41 and Cys44 are also disulfide-bonded.

In vivo redox states of some of the DsbB constructs were examined by directly dissolving TCA-precipitated proteins in SDS-AMS solution (Figure 1, lanes 7–9). DsbB[CCSS] exhibited the same mobility as the oxidized DsbB (Figure 1, lane 9), whereas DsbB[SSCC] migrated at the position of red2 (Figure 1, lane 8). Thus, the Cys41–Cys44 disulfide bond is preserved in the absence of Cys104 and Cys130, but the Cys104–Cys130 disulfide bond is not formed effectively in the absence of the other disulfide bond.

Cys104 is required for the formation of the DsbA–DsbB complex under respiration-deficient conditions

Removal of *p*-hydroxybenzoate (PHB) from the culture of the *ubiA menA* double mutant strain (Wallace and Young, 1977) led to the accumulation of a DsbA–DsbB

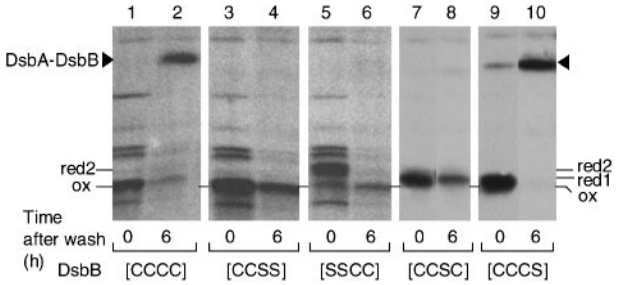

Fig. 2. Cys104 of DsbB is essential for the formation of the DsbA–DsbB complex after deprivation of quinones. Strain TA162 (*ubiA420 menA401 dsbB*::*kan*) was transformed with one of the following plasmids: pSS51 (DsbB[CCCC]; lanes 1 and 2), pTAK10 (DsbB[CCSS]; lanes 3 and 4), pTAK8 (DsbB[SSCC]; lanes 5 and 6), pSS55 (DsbB[CCSC]; lanes 7 and 8) or pSS56 (DsbB[CCCS]; lanes 9 and 10). Cells were grown at 37°C in buffered L-glucose medium supplemented with PHB. Samples for lanes 1, 3, 5, 7 and 9 were withdrawn at an exponential phase. The remaining cultures were centrifuged and cells were washed with PHB-free medium for further growth in the absence of PHB for 6 h. All samples were mixed with TCA to precipitate proteins, which were then washed with acetone and dissolved in SDS-Tris–HCl-AMS solution. Following SDS–PAGE in the absence of any reducing agent, proteins were visualized by Western blotting using anti-Myc antibodies.

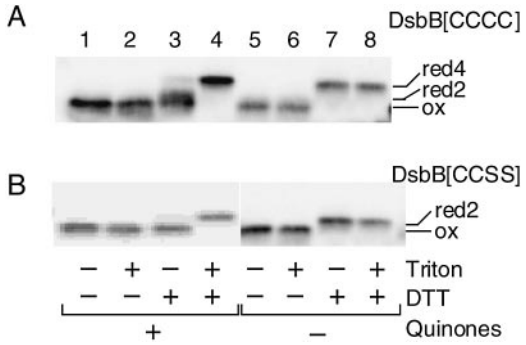

Fig. 3. Respiratory chain-dependent DTT resistance of the Cys41–Cys44 disulfide bond in membrane-integrated DsbB. Strains TA164 (*dsbB*::*kan*; lanes 1–4) and TA162 (*ubiA420 menA401 dsbB*::*kan*; lanes 5–8), each carrying pSS51 (DsbB[CCCC]) (**A**) or pTAK10 (DsbB[CCSS]) (**B**), were grown in buffered L-glucose medium. For the latter strain, PHB was included initially and then removed to deplete quinones; bacteria were grown in the absence of PHB for 12 h (lanes 5–8). Cultures were chilled on ice and centrifuged to pellet down the cells, which were then disrupted by sonication. Membrane fractions were prepared by differential centrifugations, and incubated at 0°C for 10 min with (lanes 3, 4, 7 and 8) or without (lanes 1, 2, 5 and 6) 20 mM DTT, in the presence (lanes 2, 4, 6 and 8) or absence (lanes 1, 3, 5, 7 and 9) of 1% Triton X-100. Proteins were then subjected to TCA precipitation, AMS modification and SDS–PAGE. DsbB proteins were visualized with Western blotting using anti-Myc antibodies.

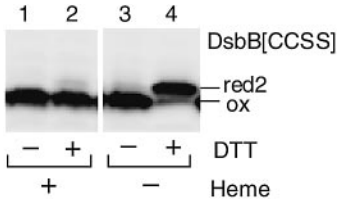

Fig. 4. Depletion of heme renders the Cys41–Cys44 disulfide bond DTT sensitive. The wild-type strain (LE392; lanes 1 and 2) and the *hemA* mutant strain (LE392, *hemA*::*kan*; lanes 3 and 4), each carrying pTAK10 (DsbB[CCSS]), were grown in buffered L-glucose medium. For the latter culture, 5-aminolevulinic acid was included initially and then removed to deplete heme (Kobayashi *et al*., 1997); bacteria were grown in the absence of 5-aminolevulinic acid for 12 h (lanes 3 and 4). Cultures were chilled on ice and centrifuged to pellet down the cells, which were then suspended in 10 mM Tris–HCl buffer pH 8.1 supplemented with or without 20 mM DTT, as indicated, and incubated at 0°C for 10 min. Whole-cell proteins were then subjected to TCA precipitation, AMS modification and SDS–PAGE. DsbB was visualized with Western blotting using anti-Myc antibodies.

complex (Kobayashi *et al*., 1997; see also Figure 2, lane 2). We examined the Cys/Ser mutational effects on this complex formation. DsbB[CCSS] did not form any DsbA–DsbB complex (Figure 2, lanes 3 and 4). DsbB[SSCC] became less abundant after quinone deprivation (Figure 2, lane 6). Although the exact mechanism of this reduction in amount is unknown, this mutant DsbB protein may somehow be destabilized under the quinone-deprived conditions. Longer exposure detected a trace of DsbB [SSCC]–DsbA complex after quinone deprivation (data not shown). Among the single Cys/Ser substitution mutants, DsbB[CCSC] was unique in that it was totally defective in the complex formation (Figure 2, lanes 7 and 8). In contrast, DsbB[CCCS] formed the complex at normal or somewhat increased efficiency (Figure 2, lanes 9 and 10). DsbB[CSCC] and DsbB[SCCC] formed some complex (data not shown), but the results with these proteins were complicated by the formation of an intramolecular disulfide bond for DsbB[CSCC] (Kishigami and Ito, 1996) and by the reduction of Cys104 and Cys130 for DsbB[SCCC]. The results presented above indicate that Cys104 is absolutely required for the formation of the DsbA–DsbB complex. Thus, the DsbA–DsbB complex formed when quinones are depleted (Kobayashi *et al*., 1997) is similar to that formed when Cys33 of DsbA is absent, in that they both involve the Cys104 residue of DsbB (Guilhot *et al*., 1995; Kishigami *et al*., 1995b; Kishigami and Ito, 1996).

The Cys41–Cys44 disulfide bond in the membrane-integrated DsbB cannot be reduced by treatment with DTT

As already shown, when cellular proteins were first denatured and then reduced with DTT, DsbB[CCCC] was modified with AMS to the red4 form (Figure 1, lane 2). In contrast, when the cells expressing DsbB[CCCC] were treated directly with DTT (which is membrane permeable), DsbB was only reduced to the red2 state, and DsbB without any molecular tag behaved similarly to the His_6-Myc-tagged protein (data not shown). The above-mentioned property of DsbB was found to be preserved even in the isolated membrane vesicles prepared by sonication and centrifugations. As shown in Figure 3A (lane 3), DTT treatment of the membrane fraction only converted DsbB[CCCC] from the ox isoform to the red2 isoform. This was true whether DTT was added before sonication or after isolation of the membranes. In striking contrast, DTT treatment of a Triton X-100-solubilized membrane preparation produced DsbB[CCCC] that was fully reducible with DTT to give the red4 mobility upon AMS modification (Figure 3A, lane 4).

The DTT sensitivity of DsbB[CCSS] was examined similarly. This protein was found to be totally refractory to reduction with DTT, either in intact cells (see Figure 4, lane 2) or in isolated membrane vesicles (Figure 3B, lane 3). In contrast, it was readily reduced when DTT treatment was performed in the presence of Triton X-100 (Figure 3B, lane 4). These results, taken together, establish that the

Cys41–Cys44 disulfide bond of the membrane-integrated DsbB protein is in such a state that it is never reduced by a reducing agent.

The Cys41–Cys44 disulfide bond is endowed with DTT resistance by the functional respiratory chain

We constructed a *ubiA420 menA401 dsbB*::*kan* triple mutant strain and transformed it with a plasmid encoding DsbB[CCCC] or DsbB[CCSS]. Cells were grown in the absence of PHB (Wallace and Young, 1977) for 12 h to deplete ubiquinone and menaquinone, and then examined for the reducibility of DsbB. It was found that DTT treatment of the DsbB[CCCC]- and the DsbB[CCSS]-expressing cells caused these proteins to migrate at the red4 and the red2 position, respectively, after AMS modification (data not shown). We prepared membrane vesicles from the quinone-depleted cells. From the results already presented, it was expected that DsbB[CCCC] would be in the form of a DsbA–DsbB complex under the quinone-deprived conditions, which we indeed observed after TCA treatment of the culture. However, we detected substantial amounts of the uncomplexed DsbB[CCCC] protein in the membrane (Figure 3A, lanes 5–8). We pointed out previously that redox-active proteins can be subject to artificial redox reactions if cells are disrupted under non-denaturing conditions (Kishigami *et al*., 1995a). Thus, after sonication, the DsbA–DsbB complex may have met some reducing factor from the cytosol (such as thioredoxin) that cleaved the disulfide linkage during sample manipulations. In any case, the DsbB[CCCC] protein thus detected was reduced readily to the red4 state by treatment with DTT (Figure 3A, lane 7).

The membrane-integrated DsbB[CCSS] was also susceptible to DTT reduction in the absence of quinones (Figure 3B, lane 7). The results for DsbB[CCSS] in the quinone-depleted membrane were less complicated, since this mutant protein did not form any complex with DsbA (see Figure 2). We also examined the reducibility of DsbB[CCSS] in heme-depleted cells. Whereas DsbB[CCSS] in the control cells was DTT resistant (Figure 4, lane 2), that in the heme-depleted cells was reduced upon incubation with DTT (Figure 4, lane 4). Thus, the loss of DTT resistance is not a unique consequence of quinone deprivation. The experiments presented so far revealed a striking coupling between the respiratory function and the non-reducibility of the Cys41–Cys44 disulfide bond of DsbB. This coupling is lost after detergent solubilization of the membrane.

Oxygen is required for DTT resistance of the Cys41–Cys44 disulfide bond

The DTT resistance of Cys41–Cys44 should have a structural and/or functional basis. For instance, the Cys41–Cys44 region of DsbB may be masked or buried in a respiration-dependent manner. Alternatively, the effect could be more functional, such that this pair of cysteines are subject to a very strong oxidation mechanism coupled with respiration; whenever they are reduced, they are oxidized back immediately. If this is the case, the DTT resistance will require oxygen. We examined whether removal of the gaseous oxygen from a membrane preparation renders DsbB reducible with DTT. A membrane fraction from DsbB[CCCC]-expressing cells was subjected to an air to N_2 exchange in the presence of DTT. It was found that DsbB[CCCC] became completely reduced to the red4 form (Figure 5, lane 1). The control sample that was exposed to DTT for the same length of time without N_2 exchange gave only the red2 form of this protein (Figure 5, lane 3). These results indicate that the air oxygen is required to keep the Cys41–Cys44 disulfide bond resistant to DTT.

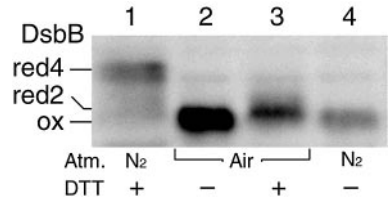

Fig. 5. Oxygen is required for DTT resistance of the Cys41–Cys44 disulfide bond. Cells of TA164 (*dsbB*::*kan*) carrying pSS51 (DsbB[CCCC]) were grown in L-glucose medium to an exponential phase, chilled on ice and harvested by centrifugation. They were then disrupted by sonication for preparation of a membrane fraction by differential centrifugations. Membranes were suspended in 10 mM Tris–HCl buffer pH 8.1 containing 1 mM phenylmethylsulfonyl fluoride and divided into four portions in microfuge tubes. Two of them (for lanes 1 and 3) received 20 mM DTT. One pair of samples (for lanes 1 and 4), with and without DTT, were sealed with plastic film and subjected to bubbling with N_2 gas through a needle while another needle allowed outflow of the internal gasses. Air to N_2 gas exchange was allowed for 30 min on ice, and incubation continued for another 30 min with complete sealing. Another pair of samples (for lanes 2 and 3) were left for 60 min without any treatment. Whole-cell proteins were then subjected to TCA precipitation, AMS modification and SDS–PAGE. DsbB was visualized with Western blotting using anti-Myc antibodies.

Discussion

Our systematic examinations of the redox states of cysteine residues of DsbB revealed that DsbB has two disulfide bonds under normal conditions. The two pairs of cysteines have contrasting properties. The C-terminally located disulfide bond between Cys104 and Cys130 is unstable by itself and requires the presence of the N-terminally located disulfide bond formed between Cys41 and Cys44. This is consistent with our previous proposal (Kishigami and Ito, 1996) that the Cys41–Cys44 disulfide bond oxidizes Cys104 and Cys130. Alternatively, the lack of the N-terminal disulfide bond causes a structural or electrostatic change to destabilize the Cys104–Cys130 disulfide bridge.

The C-terminal pair of cysteines, which are separated by 25 amino acid residues, interact with the thioredoxin-like Cys30–Pro–His–Cys33 motif of DsbA, and Cys104 is the crucial residue that initiates this interaction by forming an intermolecular mixed disulfide with Cys30 of DsbA (Guilhot *et al*., 1995; Kishigami *et al*., 1995b). The proposed intramolecular oxidation of Cys104 and Cys130 also involves interaction of this pair of cysteines with the CXXC motif, Cys41–Val–Leu–Cys44, of DsbB. It is conceivable that Cys104 is crucial in the latter reaction as well.

Two different conditions give rise to an accumulation of a disulfide-linked complex between DsbA and DsbB. One is the absence of Cys33 of DsbA. This was interpreted

to indicate that the reactive Cys30 residue of DsbA first reacts with Cys104 of DsbB as an intermediate of the DsbA reoxidation reaction, but this intermediate cannot be resolved because of the absence of Cys33 of DsbA (Guilhot *et al*., 1995; Kishigami *et al*., 1995b; Kishigami and Ito, 1996). Another condition that leads to the formation of a DsbA–DsbB complex is the deficiency in the respiratory chain (Kobayashi *et al*., 1997). We have shown here that Cys104 of DsbB is the residue that forms the complex with DsbA under the latter conditions as well.

Why does the absence of the respiratory function result in the accumulation of the DsbA–DsbB complex? It may be rationalized that this complex is an energetically favored state for the DsbA–DsbB system without the input of oxidizing power from the respiratory chain. DsbB with reduced Cys104 and Cys130 will be formed as the Cys104–Cys130 disulfide is donated to DsbA (Kishigami and Ito, 1996), and its life span may be extended in the absence of the respiratory function to allow a reverse reaction. Thus, Cys104 will attack the DsbA disulfide to make the intermolecular bridge. Our previous results showing that DsbB was reduced transiently before the formation of the DsbA–DsbB complex and that the binary complex formation preceded the reduction of the bulk of DsbA (Kobayashi *et al*., 1997) were consistent with this model. Although DsbA in the DsbB[CCCS]-expressing cells will be largely reduced (because this mutant DsbB is non-functional; Kishigami and Ito, 1996), the complex formation by the above mechanism may still be possible using a small fraction of DsbA molecules that remain oxidized (DsbA exists in excess over DsbB). It was noted indeed that a substantial amount of DsbB[CCCS]–DsbA complex was formed even under respiration-proficient conditions, when the mutant DsbB was co-expressed with wild-type DsbB that provided oxidized DsbA (T.Kobayashi, unpublished results). A contribution of low molecular weight disulfide molecules present in the medium is also possible.

We found that the Cys41–Cys44 disulfide bond of DsbB has a peculiar property in that it is never reduced even in the presence of DTT. This property is executed only when DsbB is in the membrane-integrated state. It is also lost in the absence of quinones or heme, indicating a coupling with the respiratory chain. The respiratory chain connection with the CXXC motif of DsbB is specific, since the other set of cysteines in the same protein is readily reducible even in the presence of the respiratory chain. DsbA itself, while kept oxidized in the periplasm, is susceptible to reduction by a reduced substrate protein and also by artificial addition of DTT to the medium (our unpublished results). Thus, redox states of different CXXC motifs of Dsb proteins are controlled specifically and differently. One extreme is that in DsbC, which is reduced in the steady state (Joly and Swartz, 1997; Rietsch *et al*., 1997), and the other extreme is Cys41–Cys44 of DsbB, which cannot be reduced even artificially. It will be interesting to determine the redox states of the similar motif in DsbD.

Several possible mechanisms may be considered for the DTT resistance of DsbB. First, it may be due to inaccessibility of DTT to the Cys41–Cys44 region of DsbB. For instance, some protein factor may mask the region, or, alternatively, this region could be shielded conditionally in the lipid phase of the membrane. In either case, the respiratory function must be required for the sequestration of Cys41–Cys44. It is not easy, in this case, to imagine how the sequestration of Cys41–Cys44 activates the DsbB function. The second possibility may be that this disulfide bond is intrinsically very strong. In other words, its standard redox potential is such that it is even more reducing than that of DTT_{red}/DTT_{ox}. However, it seems difficult to reconcile this possibility with the fact that the DTT resistance is only executed in the presence of oxygen and the respiratory chain. Finally, even though the Cys41–Cys44 pair does not have extreme thermodynamic properties by itself, it is maintained almost completely oxidized due to the coupling to the highly oxidizing O_2–water couple via the respiratory chain. Thus, whenever Cys41 and Cys44 are reduced, they are reoxidized immediately. Our observation that oxygen is required for the DTT resistance indicates that the last mechanism is actually operating.

Since the directionality of the redox reactions involving the DsbB CXXC motif is governed by the respiratory chain and oxygen, they should be exclusively oxidizing. In this sense, DsbB can be regarded as a DTT-oxidizing enzyme using oxygen as an ultimate electron acceptor. These considerations suggest that DsbB is involved in the recently reported observation that low ubiquinone content causes the *E.coli* cell's hypersensitivity to thiol reagents (Zeng *et al*., 1998). The respiratory chain- and oxygen-dependent protection of Cys41–Cys44 from reduction is very strong, since sonication in the presence of 20 mM DTT as well as 5 M urea did not reduce DsbB[CCSS] (data not shown).

Bader *et al*. (1998) reported recently that membrane vesicles with wild-type DsbB protein are capable of oxidizing reduced DsbA catalytically *in vitro*, and this reaction is oxygen dependent. In view of our results, we believe that this oxygen dependence is via the respiratory chain function. The DTT resistance of DsbB in the membrane vesicles seems to be an *in vitro* manifestation of the actively functioning respiratory chain, providing a simple assay for the coupling between respiratory chain and DsbB. It is conceivable that a specific protein factor mediates this coupling, and we currently are attempting to identify such a factor using biochemical and genetic approaches.

Materials and methods

Escherichia coli strains

The *E.coli* strains AN384 (*ubiA420 menA401*) and its *ubiA*$^+$ *menA*$^+$ counterpart, AN387, have been described (Wallace and Young, 1977). TA162 (*ubiA420 menA401 dsbB*::*kan5*) and TA164 (*dsbB*::*kan5*) are derivatives of AN384 and AN387, respectively, into which the *dsbB*::*kan5* mutation (Bardwell *et al*., 1993) has been introduced by P1 transduction. Strain H500, carrying the *hemA*::*kan* mutation, and its *hemA*$^+$ parent (LE392) have been described previously (Nakayashiki *et al*., 1995).

Plasmids encoding DsbB and its variants

In this study, we examined plasmid-encoded DsbB-His_6-Myc (Kishigami and Ito, 1996) and its derivatives with Cys/Ser substitution mutation(s) at residues 41, 44, 104 and 130, in various combinations. They are indicated by the four character notations in brackets, in which C and S indicate whether the residues 41, 44, 104 and 130 (in this order) are Cys or Ser. For example, DsbB[CCSS] indicates a DsbB-His_6-Myc mutant with Cys104→Ser and Cys130→Ser mutations.

Plasmids pSS51 (encoding DsbB[CCCC]), pSS53 (DsbB[SCCC]),

pSS54 (DsbB[CSCC]), pSS55 (DsbB[CCSC]) and pSS56 (DsbB[CCCS]) have been described previously (Kishigami and Ito, 1996). pTAK8, pTAK10 and pTAK18 encoded DsbB[SSCC], DsbB[CCSS] and DsbB[CSSS], respectively. The *dsbB-his*$_6$*-myc* inserts in pTAK8 and pTAK10 were constructed first from the *dsbB41S* and the *dsbB104S* derivatives of pSS43 (Kishigami and Ito, 1996), with mutagenic primers, 5′-CCTAGCGTGCTCTCTATTTATGAACG-3′ (for Cys44→Ser substitution) and 5′-CCGTTTGCCACCTCTGATTTT-ATGG-3′ (for Cys130→Ser substitution), respectively, and using the QuikChange Site-Directed Mutagenesis Kit from Stratagene. They were then recloned into pSS52 (Kishigami and Ito, 1996). For construction of pTAK18, a 2.8 kb *Afl*III–*Bsg*I fragment of the DsbB[CCSS] derivative of pSS43 (see above) and a 0.7 kb *Afl*III–*Bsg*I fragment of the *dsbB44S* derivative of pSS43 first were reconstructed and the *dsbB-his*$_6$*-myc* segment was then recloned into pSS52.

These plasmids had the pACYC184 replicon, and the *dsbB* derivative genes were placed under the *lac* promoter–operator. The *dsbB* genes were expressed in cells of *lac*$^+$ background in the presence of glucose and without addition of a *lac* inducer. Presumably, they were expressed as the *lac* repressor was titrated partially by the multicopy operator regions on the plasmids and under catabolite-repressed conditions. Under the respiration-deficient conditions, the plasmid-encoded DsbB-His$_6$-Myc molecules were all converted to the form of the DsbA–DsbB complex, while uncomplexed DsbA still remained in excess (data not shown). Therefore, their expression levels, relative to that of the DsbA protein, were similar to the level of the wild-type DsbB protein examined previously (Kobayashi *et al*., 1997).

Media and growth conditions

Cells were grown aerobically at 37°C with shaking, and cell growth was monitored as described previously (Kobayashi *et al*., 1997). L medium contained 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl and 1.7 mmol of NaOH per liter. Glucose was added at a concentration of 0.4%. Potassium phosphate buffer (pH 7.5) was added at a concentration of 0.09 M (referred to as 'buffered L medium') to avoid early growth cessation of the respiratory-deficient mutants (Kobayashi *et al*., 1997). PHB was added at a concentration of 1 mM for growing cells with the *ubiA420* mutation (Wallace and Young, 1977). For ubiquinone deprivation, cells were pelleted down by centrifugation, washed with the same medium without PHB, pelleted again and grown in the PHB-free medium for 6–12 h. When necessary, cultures were diluted with pre-warmed medium of the same composition. Depletion of heme was achieved similarly by removing 5-aminolevulinic acid from the culture of the *hemA* mutant (Kobayashi *et al*., 1997).

Preparation of membrane fractions

Cultures were chilled on ice and centrifuged at 4°C to pellet down the cells, which were suspended in 10 mM Tris–HCl pH 8.1 containing 1 mM phenylmethylsulfonyl fluoride, and disrupted by three 20 s bursts of sonication at 0–4°C (Heat Systems Ultrasonicator). Cell debris were removed by centrifugation at 9000 *g* for 5 min, and the supernatant was centrifuged at 530 000 *g* for 20 min at 4°C (Hitachi Micro Ultra-centrifuge). The membrane pellets were resuspended in the same buffer.

Determination of redox states of DsbB

Redox states of DsbB *in vivo* were determined by the same method as described previously for DsbA (Kobayashi *et al*., 1997). Thus, TCA (final concentration 5%) was added directly to a culture to denature and precipitate whole-cell proteins, which were then collected by centrifugation, washed with acetone and dissolved in 1% SDS–100 mM Tris–HCl (pH 7.5) containing 20 mM AMS by agitating at room temperature for 30 min, followed by incubation at 37°C for 10 min. Proteins were then separated by 12.5% SDS–PAGE without using any reducing agent (boiling in SDS was avoided), and electrophoretically blotted onto an Immobilon membrane filter (Millipore). The filter was then decorated with anti-c-Myc (A-14) rabbit polyclonal antibodies (Santa Cruz Biotechnology), and the DsbB-His$_6$-Myc proteins were visualized using the ECL detection kit (Amersham) and a Luminescence Image Analyzer (LAS-1000, Fuji Film). DsbB in the isolated membrane vesicles was analyzed similarly after TCA precipitation.

Electrophoretic separation of DsbB with different numbers of AMS modifications was verified by examining a series of Cys/Ser substitution mutants of DsbB-His$_6$-Myc (see Figure 1). To achieve uniform reduction of DsbB cysteines, whole-cell proteins were denatured and precipitated with TCA as described above, washed with acetone and resuspended in 10 mM Tris–HCl pH 8.1 containing 100 mM DTT. After incubation at room temperature for 20 min, TCA was added again and protein precipitates were recovered by centrifugation and processed for AMS modification as described above.

DTT treatment of membranes and cells

Typically, samples in neutral pH buffers were incubated with 20 mM DTT at 0°C for 10 min (unless otherwise specified). Under these conditions, ordinary proteins with accessible disulfide bonds are reduced completely. Samples were then TCA precipitated and processed for AMS modification as already described.

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