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The DsbA-DsbB system affects the formation of disulfide bonds in periplasmic but not in intramembraneous protein domains

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The DsbA and DsbB proteins of *Escherichia coli* are involved in facilitating the formation of disulfide bonds in periplasmic proteins. Here, we show that the rate of formation of a disulfide bond in the periplasmic domain of the inner membrane protein leader peptidase is reduced in *dsbA* and *dsbB* strains, whereas the rate of formation of a disulfide bond engineered into the membrane embedded domain of the same protein is completely unaffected by these mutations. We conclude that the Dsb proteins do not facilitate the formation of intramembraneous disulfides.

Membrane protein; Disulfide; Protein export; Leader peptidase

1. INTRODUCTION

The formation of disulfide bonds in periplasmic *E. coli* proteins has recently been shown to be facilitated by the DsbA and DsbB proteins [1–5]. DsbA is a thioredoxin-like periplasmic protein that apparently can oxidize disulfides in nascent extra-cytoplasmic proteins, whereas DsbB is an integral inner membrane protein that is thought to oxidize the reduced form of DsbA. So far, the Dsb proteins have only been shown to act on periplasmic and outer membrane proteins, and nothing is known about their possible effects on disulfide formation in integral inner membrane proteins.

One of the best characterized *E. coli* inner membrane proteins is leader peptidase (Lep), an enzyme that removes signal peptides from secretory proteins (see [6,7] for reviews). Lep has the topology shown in Fig. 1 with two transmembrane segments (H1, H2), a short cytoplasmic loop (P1), and a large periplasmic domain (P2) that contains the active site [8–10]. We have recently shown that properly located cysteines can form disulfide linkages between the H1 and H2 transmembrane segments [11], and now report on the effects of DsbA and DsbB on disulfide formation both between H1 and H2, and between Cys^{170} and Cys^{176} in the large periplasmic P2 domain. We find that the rate of formation of the Cys^{170}–Cys^{176} disulfide is markedly reduced in the *dsb* strains as compared to a wildtype strain, whereas formation of the intramembraneous Cys^{4}–Cys^{79} disulfide linking H1 and H2 is unaffected by the *dsb* mutations.

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2. MATERIALS AND METHODS

2.1. *Enzymes and chemicals*

Iodoacetamide was from Sigma. All DNA-modifying enzymes were from Promega and Pharmacia (T7 DNA polymerase).

2.2. *Strains and plasmids*

Leader peptidase mutants were expressed from the pING1 plasmid [12] in *E. coli* strains JCB570 (MC1000 *phoR* zih12::Tn*10*), JCB571 (MC1000 *phoR* zih12::Tn*10* *dsbA*::kan1), and JCB752 (MC1000 *phoR* zih12::Tn*10* *dsbB*::kan5) [1,2].

2.3. *DNA techniques*

Site-specific mutagenesis was performed according to the method of Kunkel [13], as modified by Geisselsoder et al. [14]. All mutants were confirmed by DNA sequencing of single-stranded M13 DNA using T7 DNA polymerase. Cloning into the pING1 plasmid was performed as described [15].

2.4. *Assays for disulfide bond formation*

Cells were grown overnight at 37°C in M9 minimal medium supplemented with ampicillin (100 μg/ml), 0.5% fructose, and all amino acids except methionine and cysteine, were backdiluted into the same medium (25 μl cells into 0.5 ml medium), and grown for an additional 3.5–4 h at 37°C. Expression of the mutant leader peptidase constructs was induced by addition of arabinose (2% final concentration) and incubation for 5 min. The culture was split in two just before induction with arabinose. One sample was left untreated; to the other, iodoacetamide (20 mM final concentration) was added 30 s before the [^{35}S]Met labeling in order to prevent disulfide bond formation. Both samples were then labeled with 30 μCi [^{35}S]Met for 2 min and transferred to microcentrifuge tubes. The IAA-untreated sample was incubated with iodoacetamide (20 mM final concentration) on ice for 10 min to block all free sulfhydryls. Samples were precipitated by addition of trichloroacetic acid (10% final concentration), washed with acetone, resuspended in 10 mM Tris/2% SDS, and immunoprecipitated with Lep antiserum. After resuspending in sample buffer lacking DTT, the samples were heated to 70°C for 5 min and analyzed by non-reducing SDS-PAGE (10% separating gel) and autoradiography.

The effect on disulfide bond formation of glutathione was checked by adding oxidized glutathione (6.5 mM final concentration) when the cells were backdiluted after the overnight incubation, and then proceeding as above.

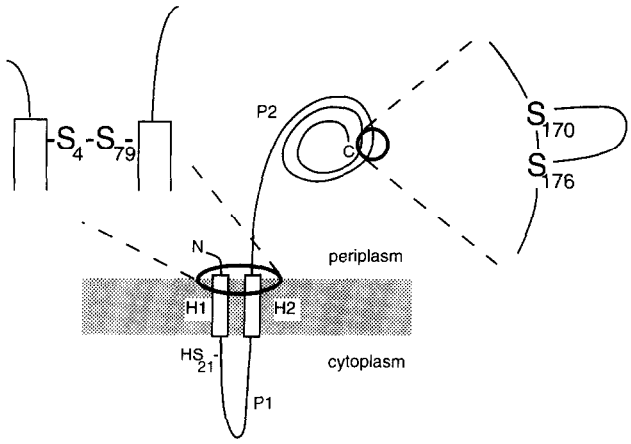

Fig. 1. Orientation of leader peptidase in the inner membrane and location of the disulfide bonds discussed in the text. Cys^{21} at the cytoplasmic end of the first transmembrane segment is also shown.

3. RESULTS

3.1. *Formation of the Cys^{170}–Cys^{176} disulfide in the periplasmic domain of Lep is affected by DsbA and DsbB*

Lep has three cysteines: Cys^{21} at the cytoplasmic end of the H1 transmembrane segment, and Cys^{170} and Cys^{176} in the periplasmic P2 domain, Fig. 1. We assayed for the formation of disulfides by labeling one-half of a culture of the wildtype strain JCB570 induced for high-level synthesis of Lep with [^{35}S]Met for 2 min and then treating with IAA to trap any remaining free sulfhydryls, while the other half was first treated with IAA for 30 s to prevent all disulfide formation prior to [^{35}S]Met labeling. The two samples were then immunoprecipitated with Lep antiserum and analyzed by SDS-PAGE under non-reducing conditions. As seen in Fig. 2, when IAA is added before the [^{35}S]Met a more slowly migrating form of the protein is produced (compare lanes 1 and 2), suggesting the formation of a disulfide bond in the absence of IAA. When Cys^{170} is mutated to Ser only the slow-moving form is seen (lanes 3 and 4), thus establishing the presence of a disulfide between residues 170 and 176. We note that this disulfide is not needed for the enzymatic activity, as the Ser170 mutant is as active as the wildtype protein [16]. As a control, the formation of the two disulfides in the PhoA protein [1] was similarly analyzed; again, when IAA was added prior to [^{35}S]Met labeling no disulfide formation was seen (data not shown).

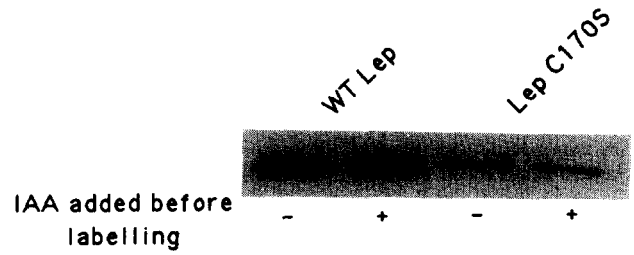

Fig. 2. Cys^{170} of wild type leader peptidase rapidly forms a disulfide bond with Cys^{176} in a wild type strain (JCB 570). A mutant Lep molecule in which Cys^{170} is changed to Ser (C170S) does not form a disulfide bond. Synthesis of Lep molecules was induced by arabinose. Cells were labeled with [35S]Met for 2 min either in the presence (+) or absence of iodoacetamide (−). After immunoprecipitation with Lep antiserum proteins were analyzed by non reducing SDS-PAGE. The lower, more rapidly moving band represents the disulfide-bonded species.

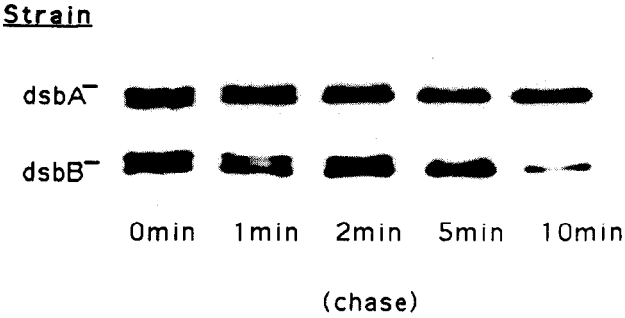

Fig. 3. The disulfide bond in wild type leader peptidase forms slowly in dsbA⁻ and dsbB⁻ strains. Synthesis of Lep molecules was induced by arabinose. Cells were labeled with [^{35}S]Met for 2 min and then chased by the addition of an excess of cold methionine for the times indicated before adding iodoacetamide to prevent further disulfide bond formation. After immunoprecipitation with Lep antiserum, proteins were analyzed by non-reducing SDS-PAGE.

When Lep was expressed in the *dsbA* and *dsbB* strains JCB571 and JCB752, the rate of formation of the Cys^{170}–Cys^{176} disulfide was strongly reduced, Fig. 3, although not as dramatically as for the PhoA control (not shown). Oxidized glutathione could substitute for DsbB but not for DsbA function (data not shown), as previously reported for disulfide formation in PhoA [2]. Thus, the Dsb-system can facilitate disulfide formation in the periplasmic domain of an inner membrane protein.

3.2. *Formation of intramembraneous disulfides between the H1 and H2 domains is not affected by DsbA and DsbB*

We have previously shown that properly positioned cysteines engineered into the H1 and H2 transmembrane segments can form disulfides in vivo [11]. One such mutant, Cys^{4}–Cys^{79} (Fig. 1), was analyzed as above (in this case, the three cysteines present in wildtype Lep had all been changed to serine). In contrast to the wildtype protein, the disulfide formed equally well in the wildtype and *dsbA* strains, and only marginally slower in the *dsbB* strain, Fig. 4. Inclusion of oxidized glutathione in the medium did not increase the fraction of disulfides formed in the wildtype strain or in the *dsbB* strain (data not shown). Similar results were obtained when other previously described H1-H2 disulfides [11] were analyzed in the *dsb* strains (data not shown). We conclude that disulfide formation between

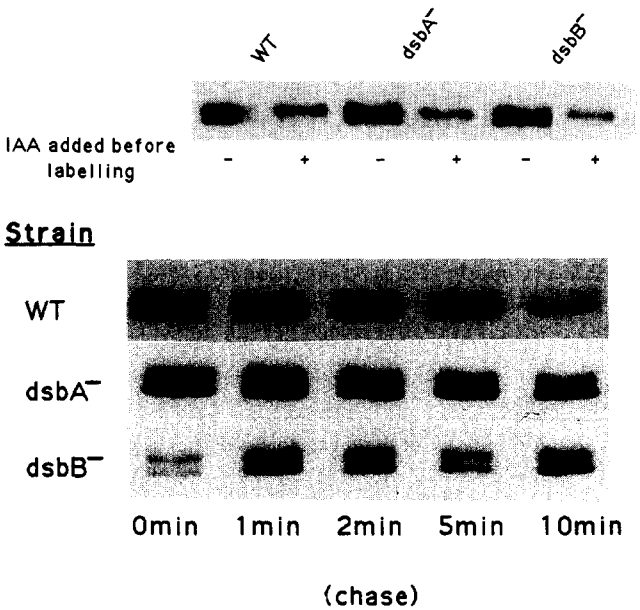

Fig. 4. Analysis of the disulfide bond formed between Cys^4 and Cys^{79} in $dsbA^-$ and $dsbB^-$ mutants. Top panel: synthesis of Lep C4,C79 was induced by arabinose. Cells were labeled for 2 min in the presence (+) or absence (−) of iodoacetamide. Proteins were analyzed by non-reducing SDS-PAGE after immunoprecipitation with Lep antiserum. The formation of the disulfide bond in Lep C4,C79 labeled in the absence of iodoacetamide is not affected in $dsbA^-$ and $dsbB^-$ mutants. Bottom panel: the different *E. coli* strains were labeled with [^{35}S]Met for 2 min after induction of Lep C4 ,C79 with arabinose. Cells were chased by the addition of an excess of cold methionine for the times indicated and then placed into iodoacetamide. Proteins were analyzed by non-reducing SDS-PAGE after immunoprecipitation with Lep antiserum.

transmembrane segments is not facilitated by the Dsb-system.

4. DISCUSSION

In this paper, we have shown that the DsbA and DsbB proteins can facilitate the formation of disulfide bonds in the periplasmic domain of an inner membrane protein, but not in the intramembraneous domain. Presumably, rapid disulfide formation is a spontaneous process in the latter case but requires the participation of a facilitator protein such as DsbA in the former. It is unlikely that the soluble DsbA protein would have access to cysteine residues in transmembrane segments, whereas one might have imagined that DsbB, which is itself an inner membrane protein, could act directly on intramembraneous cysteines. This however, does not seem to be the case; rather, DsbB most likely can only oxidize the reduced form of DsbA [2].

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