Nonconsecutive disulfide bond formation in an essential integral outer membrane protein

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The Gram-negative bacterial envelope is bounded by two membranes. Disulfide bond formation and isomerization in this oxidizing environment are catalyzed by DsbA and DsbC, respectively. It remains unknown when and how the Dsb proteins participate in the biogenesis of outer membrane proteins, which are transported across the cell envelope after their synthesis. The *Escherichia coli* protein LptD is an integral outer membrane protein that forms an essential complex with the lipoprotein LptE. We show that oxidation of LptD is not required for the formation of the LptD/E complex but it is essential for function. Remarkably, none of the cysteines in LptD are essential because either of two nonconsecutive disulfide bonds suffices for function. Oxidation of LptD, which is efficiently catalyzed by DsbA, does not involve the isomerase DsbC, but it requires LptE. Thus, oxidation is completed only after LptD interacts with LptE, an interaction that occurs at the outer membrane and seems necessary for LptD folding.

β -barrel protein | lipoprotein | protein folding

Disulfide bonds are crucial for the biogenesis of many extracytoplasmic proteins in both prokaryotes and eukaryotes (1). In Gram-negative bacteria like *Escherichia coli*, such proteins exist in the cell envelope, which is composed of two membranes that are separated by an aqueous, oxidizing compartment known as the periplasm (2, 3). In the periplasm, the oxidase DsbA introduces disulfide bonds into proteins by using the disulfide bond present in its active site at the CXXC motif; in this step, DsbA becomes reduced (4, 5). To allow the system to turn over, reduced DsbA is reoxidized by the inner membrane (IM) protein DsbB (6).

Envelope proteins containing multiple disulfide bonds can be oxidized incorrectly. Introduction of the wrong disulfide bond by DsbA is particularly problematic for proteins that require a disulfide bond between nonconsecutive Cys (7–10). When such proteins are oxidized incorrectly, the periplasmic disulfide bond isomerase DsbC rearranges their disulfide bonds to produce the final nonconsecutive configuration (11–13). In this process, DsbC becomes oxidized and it must be reduced by the IM protein DsbD to be reactivated (14).

Recently the biogenesis of the periplasmic protein PhoA, which contains two consecutive disulfide bonds, has been investigated (15). PhoA can be secreted across the IM by the Sec translocon either co- or posttranslationally. When PhoA undergoes cotranslational translocation, DsbA introduces disulfide bonds between consecutive Cys vectorially as the protein appears in the periplasm; when PhoA undergoes posttranslational translocation, disulfide bonds do not necessarily form vectorially. Because wild-type PhoA contains consecutive disulfide bonds, its biogenesis does not depend on DsbC; however, DsbC plays a role in the biogenesis of mutant PhoA proteins that contain a nonconsecutive disulfide bond.

In addition to soluble periplasmic proteins, bacterial outer membrane (OM) proteins can also contain disulfide bonds. Integral OM proteins typically span the OM as β -sheets folded in a barrel conformation. Their folding and insertion into the OM is catalyzed by the Bam complex (16–21) in conjunction with periplasmic chaperones (22). However, because of the extra complexity in their biogenesis, it remains unclear how OM proteins are oxidized.

One of the most important OM proteins, LptD (formerly Imp), contains disulfide bonds (23). LptD is essential in *E. coli* (23), and it forms a heterodimer complex with the OM lipoprotein LptE (formerly RlpB) (24, 25). The LptD/E complex is the OM component of the Lpt (LPS transport) pathway, which transports LPS from the IM to the outer leaflet of the OM (26, 27). Proper assembly of LPS at the cell surface (28) is crucial for the OM to function as a permeability barrier that protects bacteria from hydrophobic antibiotics and detergents (29).

LptD is unstable without LptE because LptD cannot be overproduced unless *lptE* is also overexpressed (24). The interaction between LptD and LptE is so strong that the complex does not dissociate even during SDS/PAGE. Mature LptD is composed of two essential domains: a soluble, 171-aa N-terminal domain of unknown function that is predicted to be in the periplasm, and a 589-aa C-terminal domain that is sufficient for complex formation with LptE in vivo and that is thought to adopt a β -barrel conformation in the OM. In addition, LptD contains four Cys at positions 31, 173, 724, and 725 of pre-LptD (positions 7, 149, 700, and 701 in mature LptD). Thus, each of the aforementioned N- and C-terminal domains contains a pair of Cys.

Evidence that LptD contains disulfide bonds stems from changes in migration through SDS-polyacrylamide gels (SDS-PAGs) caused by the presence of reducing agents (23). Furthermore, a mutant form of DsbA that traps substrates in vivo has been shown to interact directly with LptD (30). However, the number and connectivity of disulfide bonds in LptD is unknown. It is not even clear whether disulfide bonds are required for LptD function because LptD is essential but DsbA is not. Here we explore the role of disulfide bonds of LptD in vivo and present insight into the pathway for the biogenesis of the essential LptD/E complex.

Results

LptD Is a Monomer That Contains Intramolecular Disulfide Bond(s). It has been suggested that at least one of the Cys in LptD forms an intermolecular disulfide bond because oxidized LptD (LptD_{OX}) migrates slower than reduced LptD (LptD_{RED}) during SDS/PAGE (23). However, the LptD/E complex is a heterodimer (24). Given that the single Cys present in LptE cannot form a disulfide bond, because it is the site for the addition of the lipid moiety found in lipoproteins (31), any disulfide bonds in LptD must be intramolecular.

To confirm that indeed LptD_{OX} migrates slower than LptD_{RED}, we analyzed migration patterns by immunoblotting after reducing (+ β -ME) and nonreducing (– β -ME) SDS/PAGE using whole-cell

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samples that have been boiled to dissociate LptD from LptE (24). In the nonreduced samples, LptD_{OX} migrates as a single band ≈ 130 kDa (Fig. 1A, $-\beta$ -ME samples), but addition of β -ME changes its mobility to ≈ 90 kDa, the expected size of LptD_{RED} (Fig. 1A, $+\beta$ -ME samples). Thus, unlike most proteins containing intramolecular disulfide bonds, LptD_{RED} migrates faster than LptD_{OX}, a phenomenon that has been reported before (32, 33). The slower mobility of LptD_{OX} is not caused by the formation of mixed disulfides between LptD and another protein during sample preparation because the same mobility is observed when using standard trichloroacetic acid (TCA) precipitation and iodoacetamide treatments (Fig. S1).

The two additional bands around 130 kDa are not different forms of oxidized LptD, because they are present at equivalent levels in a *surA* strain (Fig. 1A). Cells lacking the periplasmic chaperone SurA (2) have greatly reduced levels of LptD (34).

Disulfide Bond Formation Is Essential for LptD Function. To investigate the number, connectivity, and role of disulfide bonds in LptD, we constructed every possible *lptD* Cys mutant allele by mutating the four Cys codons singly or in combination to Ser codons in an otherwise wild-type *lptD* gene carried on pET2342 (25). In our strains, which lack T7 polymerase, this plasmid produces LptD to levels comparable to those made from the native chromosomal locus (see below). Strains containing the mutagenized plasmids exhibit no growth defects in the presence of a wild-type *lptD* chromosomal allele, suggesting that none of the mutant LptD proteins are toxic.

Because LptD is an essential protein in *E. coli* (23), we assessed whether disulfide bonds are required for LptD function by determining whether a null Δ *lptD2::kan* allele could replace the native *lptD* chromosomal gene in strains carrying plasmids that produce one of the following: wild-type LptD (for clarity, LptD_{CCCC}), each of the four LptD triple Cys-less mutants (LptD_{CSSS}, LptD_{SCSS}, LptD_{SSCS}, and LptD_{SSSS}), or Cys-less LptD (LptD_{SSSS}). The Δ *lptD2::kan* allele could be introduced into the strain producing LptD_{CCCC} but not into those producing LptD mutant proteins that lack all or any three Cys (Table 1), demonstrating that formation of disulfide bond(s) in LptD is essential.

In some proteins, disulfide bond formation is required to drive protein folding, and misfolded mutant proteins that lack disulfide bonds are often degraded by cellular proteases (5). However, an *lptD*_{CCCC}/*lptD*_{SSSS} diploid strain contains similar amounts of LptD_{RED} (≈ 90 kDa, LptD_{SSSS}) and LptD_{OX} (≈ 130 kDa, LptD_{CCCC}) (Fig. 1B). Thus, although eliminating all Cys does not significantly destabilize LptD, it renders the protein nonfunctional.

Nonconsecutive Disulfide Bond in LptD That Bridges the N- and C-Terminal Domains of LptD Is Required for Function. Two of the

Table 1. Viability of haploid *lptD* Cys mutants in LB

Single*	Growth	Double*	Growth	Triple*	Growth	Quadruple*	Growth
CCCC	+	SSCC	–	SSSC	–	SSSS	–
SCCC	+	SCSC	+	SSCS	–		
CSCC	–	SCCC	–	SCSS	–		
CSCC	+	CSSC	–	CCCS	–		
CCSC	+	CSCS	+				
		CCSS	–				

*Amino acids present at positions 31, 173, 724, and 725 of LptD.

double Cys-less mutant proteins, LptD_{SCSC} and LptD_{CSCS}, are functional (Table 1). These proteins contain one disulfide bond each, because they show different mobility through nonreducing SDS-PAGEs but the same mobility in the presence of β -ME (Figs. 1C and 2 and Fig. S1). The difference in migration of these proteins in nonreducing conditions must be caused by the different shape/net charge that each of the denatured proteins adopts as a result of their disulfide bond (Fig. 1C and Fig. S1). These results demonstrate that although LptD contains four Cys, it only needs one of the two nonconsecutive pairs for function. Thus, the N- and C-terminal domains of LptD must be covalently bonded through either a Cys₁₇₃–Cys₇₂₅ or a Cys₃₁–Cys₇₂₄ disulfide bond.

Wild-Type LptD Is Fully Oxidized. Given that either the Cys₁₇₃–Cys₇₂₅ or the Cys₃₁–Cys₇₂₄ disulfide bond is sufficient for function, LptD_{CCCC} could exist as a mixture of each of these two species or be fully oxidized. However, the fact that LptD_{CCCC} migrates slightly faster than LptD_{SCSC} and LptD_{CSCS} in nonreducing SDS-PAGEs (Fig. 1C and Fig. S1) indicates that wild-type LptD is fully oxidized in vivo.

Moreover, we measured the content of free thiols capable of reacting with Ellman's reagent (35) in a sample of purified LptD_{CCCC}/LptE complex. Purified LptD_{CCCC} has the same electrophoretic mobility on a nonreducing SDS-PAGE as LptD in cells, indicating that purified LptD has the same oxidation state. The sample contained 3.94 ± 0.62 free thiol per LptD protein equivalent if disulfide bonds were reduced, but only 0.38 ± 0.26 if they were not. Therefore, LptD is fully oxidized in the LptD/E complex. Because for function LptD must contain either the Cys₁₇₃–Cys₇₂₅ or the Cys₃₁–Cys₇₂₄ disulfide bond, we conclude that fully oxidized LptD_{CCCC} has both of these bonds between nonconsecutive Cys.

Defects in Oxidation in *lptD* Single Cys-less Mutants Are Suppressed in Double Cys-less Mutants. Because either the Cys₁₇₃–Cys₇₂₅ or

Fig. 1. LptD is fully oxidized but disulfide bonds are not required for LptD stability. (A) Whole-cell samples from wild type (wt, NR754) and Δ *surA* (NR1215) were boiled in the absence or presence of β -ME and subjected to LptD immunoblot analysis. Reduced and oxidized LptD (LptD_{RED} and LptD_{OX}, respectively) and an unidentified 55-kDa IM protein are marked (23). Size (in kilodaltons) of molecular mass markers are shown on the left. (B) Samples from NR754 (pET2342/*lptD*_{SSSS}), which produces LptD_{CCCC} (marked as CCCC) and LptD_{SSSS} (marked as SSSS), were subjected to nonreducing SDS/PAGE and immunoblotting using anti-LptD antiserum. (C) Samples from NR754 Δ *lptD* (pET2342/*lptD*_{CCCC}), NR754 Δ *lptD* (pET2342/*lptD*_{SCSC}), and NR754 Δ *lptD* (pET2342/*lptD*_{CSCS}) were analyzed by nonreducing SDS/PAGE followed by LptD immunoblotting. Different LptD proteins adopt different shapes (Right) when denatured because of the connectivity of their disulfide bonds. The double and solid lines represent the N- and C-terminal domains of LptD, respectively.

Fig. 2. Oxidation patterns of functional LptD Cys mutant proteins. Whole-cell samples from haploid NR754 Δ *lptD* (pET2342/*lptD*_{wt} or mutant) strains expressing wild-type (lane marked CCCC) or LptD Cys mutant proteins (Cys content marked above lanes) were subjected to nonreducing (Lower) and reducing (Upper) SDS/PAGE followed by LptD immunoblotting. Samples were obtained from cells grown to OD₆₀₀ $\approx$ 0.8. Size (in kilodaltons) of molecular mass markers are shown on the left. Asterisks mark bands in the doublet present in the CCCC lane.

the Cys₃₁–Cys₇₂₄ disulfide bond is sufficient for LptD function, all haploid *lptD* single Cys-less mutants should be viable. Paradoxically, the *lptD*_{CSCC} mutant is not (Table 1). Apparently the lethality is caused by inappropriate oxidation because the C173S substitution is suppressed by C725S (Table 1, compare LptD_{CSCC} and LptD_{CSCS}). For clarity, the significance of this finding follows in sections below.

In agreement with the above results on connectivity and requirement for function, all of the viable *lptD* single Cys-less mutants produce either the Cys₃₁–Cys₇₂₄ or Cys₁₇₃–Cys₇₂₅ disulfide-bonded species on nonreducing SDS-PAGEs, depending on which Cys pair each particular protein contains (Fig. 2, $-\beta$ -ME). It is apparent, however, that LptD_{SCCC} and LptD_{CCCC} are not properly oxidized.

In addition to the small amounts of Cys₁₇₃–Cys₇₂₅ disulfide-bonded species detected, the *lptD*_{SCCC} mutant also contains LptD species that migrate as LptD_{RED} (Fig. 2, $-\beta$ -ME). This defect is less significant in samples from overnight cultures (Fig. S2). Interestingly, this apparent oxidation defect is also observed, although less severely, in LptD_{SCSC}, suggesting that the C724S substitution partially suppresses the defect caused by the C31S substitution.

LptD_{CCCC} migrates as a doublet (Fig. 2, $-\beta$ -ME, asterisks). The top band of this doublet corresponds to a species containing the functional Cys₃₁–Cys₇₂₄ disulfide bond, whereas the bottom band migrates as the Cys₁₇₃–Cys₇₂₅ disulfide-bonded species. However, because LptD_{CCCC} lacks Cys₇₂₅, this latter species must contain the Cys₁₇₃–Cys₇₂₄ bond. Thus, in the absence of Cys₇₂₅, Cys₇₂₄ can bond with either Cys₃₁ or Cys₁₇₃, but formation of the nonfunctional Cys₁₇₃–Cys₇₂₄ bond is favored over that of the functional Cys₃₁–Cys₇₂₄ bond. In fact, low levels of functional LptD in the *lptD*_{CCCC} mutant may be the reason why this particular strain, unlike all other viable Cys mutants, exhibits OM-permeability defects (SI Text). Remarkably, both the incorrect oxidation and permeability defects in *lptD*_{CCCC} are corrected in *lptD*_{CSCS} cells; they make much higher levels of the functional Cys₃₁–Cys₇₂₄ disulfide bond than *lptD*_{CCCC} cells (Fig. 2, $-\beta$ -ME) and are no longer hypersensitive to SDS/EDTA (SI Text). Thus, the C173S substitution suppresses defects caused by the C725S substitution.

Taken together, these genetic interactions suggest that problems in LptD oxidation caused by a missing Cys can be alleviated by eliminating the Cys that it normally pairs with.

***lptD*_{CSCC} Is Conditionally Lethal.** As stated above, in LptD proteins lacking Cys₃₁ or Cys₁₇₃, formation of the functional disulfide bond between the remaining Cys pair is defective, and this defect is suppressed in the double mutants that lack either Cys₇₂₄ or Cys₇₂₅, respectively. In other words, the defects caused by the loss of either Cys₃₁ or Cys₁₇₃ are exacerbated when both Cys₇₂₄ and Cys₇₂₅ are present. We hypothesized that perhaps the absence of either Cys₃₁ or Cys₁₇₃ leads to the formation of a Cys₇₂₄–Cys₇₂₅ disulfide bond that is detrimental because the resulting protein would not be functional. It should be noted that LptD containing only the putative Cys₇₂₄–Cys₇₂₅ bond would probably migrate as LptD_{RED} because disulfide bonds between Cys that are proximal in the primary sequence often do not alter protein mobility (15, 36).

This hypothesis predicts that both LptD_{SCCC} and LptD_{CSCC} should have similar defects. Still, cells expressing LptD_{SCCC} make enough of the functional Cys₁₇₃–Cys₇₂₅ bonded species to be viable, but cells expressing LptD_{CSCC} do not. If so, there might be growth conditions that minimize formation of the putative Cys₇₂₄–Cys₇₂₅ bond and thus allow growth of the *lptD*_{CSCC} haploid strain. Because the rich media used in experiments reported above contain oxidants (4, 6, 37), we tested functionality of LptD_{CSCC} in minimal medium.

Indeed, the haploid *lptD*_{CSCC} strain could be constructed using minimal medium. Notably, in addition to the five mutant proteins already reported as functional (Table 1), the only other Cys LptD mutant protein that supports growth is LptD_{CSCC}. The fact that every single Cys mutant is viable in minimal medium reveals that even though a disulfide bond (Cys₃₁–Cys₇₂₄ or Cys₁₇₃–Cys₇₂₅) is essential for function, there is no individually essential Cys in LptD.

The haploid *lptD*_{CSCC} strain grows in minimal medium but not in LB (Fig. S3). Clearly, LB medium contains oxidants that inhibit the growth of the haploid *lptD*_{CSCC} strain because adding the reducing agent DTT suppresses such growth inhibition, as demonstrated in a disk diffusion assay using a paper disk containing DTT placed onto a lawn of haploid *lptD*_{CSCC} cells (Fig. 3A). Growth only occurs at a narrow range of DTT concentrations, as indicated by the ring of growth around the disk. In contrast, growth of a wild-type *lptD*_{CCCC} haploid strain is unaffected by the DTT-containing disk. Thus, unlike *lptD*_{CCCC} cells, *lptD*_{CSCC} cells are sensitive to both reducing and oxidizing agents. In the zone showing inhibition of growth between the disk and the ring, the DTT concentration is too high to support growth, probably because the formation of the Cys₃₁–Cys₇₂₄ bond is not efficient in the absence of Cys₁₇₃, as demonstrated by the oxidation defects seen in *lptD*_{CSCS} cells (Fig. 2 and Fig. S2). In the zone beyond the ring, the DTT concentration is too low to counteract the oxidizing agent(s) in LB that likely promotes formation of the putative Cys₇₂₄–Cys₇₂₅ bond.

We also analyzed mobility of LptD_{CSCC} through a nonreducing SDS-PAGE using samples from cells grown in minimal medium. In whole-cell samples, the $\approx$ 90-kDa species of LptD_{CSCC} is easily detected but not the expected Cys₃₁–Cys₇₂₄ species; detecting the latter required concentrated crude OM preparations (Fig. 3B). Such growth and sample preparation does not alter the migration pattern of LptD_{CCCC} or LptD_{CSCS} with respect to those reported above (Figs. 2 and 3B). A small fraction of LptD_{CSCC} contains the functional Cys₃₁–Cys₇₂₄ bond, but the majority of the protein migrates as a $\approx$ 90-kDa band, like LptD_{RED}. These results agree with the hypothesis that the Cys₇₂₄–Cys₇₂₅ bond forms and is detrimental. Therefore, we propose that in LptD proteins lacking either Cys₃₁ or Cys₁₇₃, molecules containing the putative dead-end Cys₇₂₄–Cys₇₂₅ bond are more abundant than those containing

Fig. 3. LptD_{CSCC} is not properly oxidized and is sensitive to both oxidizing and reducing agents. (A) Lawn of $\approx 5 \times 10^7$ cells of NR754 Δ lptD (pET2342/lptD_{CSCC}) in LB top agar was placed on an LB plate with a disk containing DTT. (B) Crude OM preparations from NR754 Δ lptD (pET2342/lptD_{CCCC}), NR754 Δ lptD (pET2342/lptD_{CSCC}), and NR754 Δ lptD (pET2342/lptD_{CSCS}) grown to OD₆₀₀ ≈ 0.6 were subjected to reducing (+β-ME) and nonreducing (−β-ME) SDS/PAGE followed by LptD immunoblotting. Size (in kilodaltons) of molecular mass markers are shown on the left.

the functional bond; however, if the C-terminal nonpairing Cys is absent (i.e., in the functional double Cys-less mutants), then formation of the Cys₇₂₄–Cys₇₂₅ bond cannot occur. This would explain why the defects in the single Cys-less *lptD_{SCCC}* and *lptD_{CSCC}* mutants are suppressed in the double Cys-less *lptD_{SCSC}* and *lptD_{CSCS}* mutants, respectively.

DsbA Is Important but Not Required for the Oxidation of LptD. A mutant form of DsbA deficient in the release of its substrates interacts with LptD (30), suggesting that DsbA oxidizes LptD directly. However, if DsbA was required for disulfide bond formation in LptD, DsbA would be essential in *E. coli* because LptD must contain at least either the Cys₃₁–Cys₇₂₄ or the Cys₁₇₃–Cys₇₂₅ bond to support growth (Table 1). Yet DsbA is not essential in our strain background MC4100 (4).

Nevertheless, *dsbA* samples subjected to nonreducing SDS/PAGE show defects in LptD oxidation (Fig. 4) similar to those exhibited by LptD_{SCCC} (Fig. 2). Specifically, there is a small population of LptD that migrates as oxidized LptD_{CCCC} containing both Cys₃₁–Cys₇₂₄ and Cys₁₇₃–Cys₇₂₅ bonds, but most of the protein has the same ≈ 90 -kDa mobility as LptD_{RED} (Fig. 4). Furthermore, the levels of this latter species decrease with a concomitant increase in the levels of properly oxidized LptD in samples from overnight cultures (Figs. S2 and S4). Whether the similarities between the loss of Cys₃₁ and that of DsbA are the result of the same defect in the oxidation pathway remains to be determined.

Last, the *dsbA* mutant grows in minimal medium aerobically but not anaerobically, despite that in LB medium it can grow under both conditions. Although it is unclear whether these phenotypes are the result of defects in LptD oxidation, they demonstrate that oxidants in the media and oxygen can partially substitute for DsbA. This would explain why DsbA is not essential even though oxidation of LptD is.

DsbC Is Not Required for LptD Oxidation. Because LptD contains two nonconsecutive disulfide bonds, we investigated whether DsbC plays a role in the biogenesis of LptD by determining the oxidation status of LptD_{CCCC} in strains lacking this isomerase. The absence of DsbC does not alter the levels or oxidation pattern of LptD (Fig. 4 and Fig. S4), suggesting that DsbC is not required in LptD biogenesis.

Fig. 4. DsbA but not DsbC is involved in LptD oxidation. Samples from wild-type (NR754), *dsbA::kan* (NR1216), and *dsbC::cam* (NR1217) cells grown to OD₆₀₀ ≈ 0.8 were subjected to nonreducing (−β-ME) and reducing (+β-ME) SDS/PAGE and LptD immunoblotting. Size (in kilodaltons) of molecular mass markers are shown on the left.

LptE Is Required for Proper Oxidation of LptD. LptE is required for LptD stability, suggesting that LptE might be necessary for proper folding of LptD (24). Because LptD and LptE are targeted to the OM by different pathways, formation of the LptD/E complex must occur at the OM. Therefore, we could test whether LptD oxidation is completed before its interaction with LptE at the OM by analyzing the oxidation status of LptD in an LptE-depletion strain in which expression of *lptE* is under the control of an arabinose-inducible promoter (25).

In the presence of arabinose, LptE is produced and LptD is properly oxidized (Fig. 5). In the absence of arabinose, when LptE is not produced, LptD is not detected under nonreducing conditions. However, when these samples are treated with β-ME, as much LptD_{RED} can be detected as in samples from cells not depleted for LptE, suggesting that in the absence of LptE, LptD likely forms SDS-resistant aggregates that are sensitive to reducing agents. Therefore, proper oxidation of LptD requires LptE.

To determine whether the defect in LptD biogenesis is specific to the loss of LptE, we analyzed cells depleted for different Lpt factors, because depletion of any Lpt factor results in the same lethal envelope defects that could indirectly compromise disulfide bond formation (26, 38). An LptF/G double depletion strain (39) revealed that depletion of LptFG does not result in defective oxidation of LptD (Fig. 5), demonstrating that defects in the oxidation of LptD that occur upon depletion of LptE are specific to the loss of LptE.

Fig. 5. LptE is required for proper oxidation of LptD. The arabinose-dependent LptE-depletion strain AM689 (25) was grown in the presence (+LptE) or absence (−LptE) of arabinose. The arabinose-dependent LptFG-depletion strain NR1113 (39) was grown in the absence (−LptFG) of arabinose. Crude OM preparations were subjected to reducing (+β-ME) and nonreducing (−β-ME) SDS/PAGE followed by LptD (Upper) and LptE (Lower) immunoblotting. Size (in kilodaltons) of molecular mass markers are shown on the left.

Discussion

Disulfide bonds are essential for LptD function and, therefore, viability of *E. coli*. Using a combination of genetic and biochemical analyses that analyzed the connectivity of disulfide bonds in vivo, we have demonstrated that LptD contains two disulfide bonds between nonconsecutive Cys, but either of these bonds suffices for function. Thus, none of the four Cys in LptD are essential.

Oxidation does not seem to drive the folding of LptD, nor is it required for the formation of the LptD/E complex (24). Instead, it seems that disulfide bonds in LptD stabilize a conformation that correctly positions the N- and C-terminal domains of LptD in the periplasm. This positioning is essential for function, and it might be required for the interaction of the LptD/E complex with its substrate LPS and/or with other Lpt factors to build the transenvelope complex that likely transports LPS across the cell envelope (40).

It is widely assumed that the biogenesis of most bacterial envelope proteins containing disulfide bonds between nonconsecutive Cys depends on the disulfide isomerase DsbC. This assumption is based on a model whereby oxidation occurs vectorially while the protein is being translocated across the IM (7). Indeed, such a model has proven correct for PhoA molecules that undergo cotranslational translocation from the cytoplasm (15). However, if translocation across the IM occurs posttranslationally, oxidation of PhoA is not necessarily vectorial (15), and a significant fraction of LptD, like other OMPs, is likely translocated across the IM posttranslationally. Likewise, if oxidation occurs after some folding of the substrate has occurred, connectivity will not be determined by the primary amino acid sequence.

LptD is efficiently oxidized by DsbA, but quantities of properly oxidized LptD that are sufficient to support growth can also be formed by nonspecific oxidants in the environment. This explains why disulfide bond formation in LptD is essential in *E. coli*, yet DsbA is not. We do not know where in the cell envelope oxidation of LptD begins and how much folding, if any, of LptD occurs before DsbA oxidizes the molecule. However, the fact that proper oxidation of LptD depends on its interaction with LptE at the OM suggests that oxidation by DsbA might begin after folding of LptD brings the N- and C-terminal Cys residues into close proximity. According to this model, DsbA would form the correct disulfide bonds directly so isomerization by DsbC would not be required. An alternative model posits that DsbA interacts with LptD before LptD reaches the OM. In this model, DsbA forms incorrect disulfide bonds in LptD that are subsequently rearranged in a DsbC-independent manner.

Strikingly, proper oxidation of LptD requires complex partner LptE. Because LptD and LptE are targeted and assembled at the

OM by different pathways (SurA/BamABCDE and LolABCDE, respectively), this finding implies that oxidation of LptD is not completed until LptD reaches the OM and interacts with LptE. We propose that LptE is required for LptD folding and that at least some folding is required to properly align the Cys residues in the N terminus and C terminus to allow proper oxidation. Why LptD has two disulfide bonds when one would suffice is a question that will require a more in-depth mechanistic analysis. Perhaps this added complexity provides a molecular mechanism for the posttranslational regulation of OM biogenesis.

Materials and Methods

Bacterial Strains and Growth Conditions. All strains, plasmids, growth conditions, OM-permeability assays, and DTT sensitivity are described in *SI Materials and Methods*.

Immunoblotting. One-milliliter samples from cultures were pelleted (16,000 × g, 2 min). Pellets were resuspended in a volume (mL) equal to OD₆₀₀/10 of SDS sample buffer lacking or containing 5% (vol/vol) β-ME. Samples were boiled for 10 min, and 20 μL were subjected to electrophoresis through 10% SDS-PAGEs and immunoblotting. Rabbit polyclonal antisera against LptD (1:7,500 dilution) and LptE (1:3,000 dilution) (23, 24) and donkey ECL horseradish peroxidase conjugate anti-rabbit IgG (GE Life Sciences) (1:10,000 dilution) were used in immunoblots. Bands were visualized using the ECL antibody detection kit (Amersham Pharmacia Biotech) and Hyblot CL film (Denville Scientific).

Isolation of OM for Analysis of LptD Oxidation States. Refer to *SI Materials and Methods* for details on depletion experiments. A 500-mL culture was grown at 37 °C to OD₆₀₀ ≈ 0.5–0.6. Cells were pelleted at 5,000 × g for 20 min and resuspended in 5 mL Tris-B buffer (10 mM Tris-HCl, pH 8.0) containing 20% (wt/wt) sucrose, 1 mM phenylmethylsulfonyl fluoride (Sigma), 50 μg/mL DNase I (Sigma), and 50 mM iodoacetamide (Sigma). Cells were lysed by a single passage through a French Press (Thermo Electron) at 8,000 psi. Approximately 8 mL of cell lysate was layered onto a two-step sucrose gradient [top, 4 mL Tris-B buffer containing 40% (wt/wt) sucrose; bottom, 1 mL Tris-B buffer containing 65% (wt/wt) sucrose] and centrifuged at 260,800 × g for 16 h in a Beckman SW41 rotor in a XL-90 ultracentrifuge (Beckman). OM fragments (≈ 0.5 mL) were isolated from the 40%/65% interface by puncturing the side of the tube with a syringe. One milliliter of 20 mM Tris-HCl, pH 8.0, was added to the OM fragments to lower the sucrose concentration to below 20% (wt/wt). The OM fragments were pelleted in a microcentrifuge at 18,000 × g for 30 min and resuspended in 200–250 μL TBS containing 5 mM iodoacetamide. Protein concentration of these OM preparations were determined using Bio-Rad D_C protein assay after precipitating in 10% TCA and resolubilizing in TBS containing 2% SDS. The same amount of OM (based on protein content) for each strain was analyzed by nonreducing SDS/PAGE and immunoblotted (24).

For additional information, refer to *SI Materials and Methods*.

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- Riemer J, Bulleid N, Herrmann JM (2009) Disulfide formation in the ER and mitochondria: Two solutions to a common process. *Science* 324:1284–1287.
- Bos MP, Robert V, Tommassen J (2007) Biogenesis of the gram-negative bacterial outer membrane. *Annu Rev Microbiol* 61:191–214.
- Ruiz N, Kahne D, Silhavy TJ (2006) Advances in understanding bacterial outer-membrane biogenesis. *Nat Rev Microbiol* 4:57–66.
- Bardwell JC, McGovern K, Beckwith J (1991) Identification of a protein required for disulfide bond formation in vivo. *Cell* 67:581–589.
- Manmathambika BS, Bardwell JC (2008) Disulfide-linked protein folding pathways. *Annu Rev Cell Dev Biol* 24:211–235.
- Bardwell JC, et al. (1993) A pathway for disulfide bond formation in vivo. *Proc Natl Acad Sci USA* 90:1038–1042.
- Berkmen M, Boyd D, Beckwith J (2005) The nonconsecutive disulfide bond of *Escherichia coli* phytase (AppA) renders it dependent on the protein-disulfide isomerase, DsbC. *J Biol Chem* 280:11387–11394.
- Hiniker A, Bardwell JC (2004) In vivo substrate specificity of periplasmic disulfide oxidoreductases. *J Biol Chem* 279:12967–12973.
- Joly JC, Swartz JR (1997) In vitro and in vivo redox states of the *Escherichia coli* periplasmic oxidoreductases DsbA and DsbC. *Biochemistry* 36:10067–10072.
- Shouldice SR, et al. (2010) In vivo oxidative protein folding can be facilitated by oxidation-reduction cycling. *Mol Microbiol* 75:13–28.
- Missiakos D, Georgopoulos C, Raina S (1994) The *Escherichia coli* dsbC (xprA) gene encodes a periplasmic protein involved in disulfide bond formation. *EMBO J* 13:2013–2020.
- Shevchik VE, Condemine G, Robert-Baudouy J (1994) Characterization of DsbC, a periplasmic protein of *Erwinia chrysanthemi* and *Escherichia coli* with disulfide isomerase activity. *EMBO J* 13:2007–2012.
- Rietsch A, Belin D, Martin N, Beckwith J (1996) An in vivo pathway for disulfide bond isomerization in *Escherichia coli*. *Proc Natl Acad Sci USA* 93:13048–13053.
- Rietsch A, Bessette P, Georgiou G, Beckwith J (1997) Reduction of the periplasmic disulfide bond isomerase, DsbC, occurs by passage of electrons from cytoplasmic thioredoxin. *J Bacteriol* 179:6602–6608.
- Kadokura H, Beckwith J (2009) Detecting folding intermediates of a protein as it passes through the bacterial translocation channel. *Cell* 138:1164–1173.
- Doerrler WT, Raetz CR (2005) Loss of outer membrane proteins without inhibition of lipid export in an *Escherichia coli* YaeT mutant. *J Biol Chem* 280:27679–27687.
- Malinverni JC, et al. (2006) YfiO stabilizes the YaeT complex and is essential for outer membrane protein assembly in *Escherichia coli*. *Mol Microbiol* 61:151–164.
- Sklar JG, et al. (2007) Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in *Escherichia coli*. *Proc Natl Acad Sci USA* 104:6400–6405.
- Werner J, Misra R (2005) YaeT (Omp85) affects the assembly of lipid-dependent and lipid-independent outer membrane proteins of *Escherichia coli*. *Mol Microbiol* 57:1450–1459.

20. Wu T, et al. (2005) Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. *Cell* 121:235–245.
21. Hagan CL, Kim S, Kahne D (2010) Reconstitution of outer membrane protein assembly from purified components. *Science* 328:890–892.
22. Leverrier P, Vertommen D, Collet JF (2010) Contribution of proteomics toward solving the fascinating mysteries of the biogenesis of the envelope of *Escherichia coli*. *Proteomics* 10:771–784.
23. Braun M, Silhavy TJ (2002) Imp/OstA is required for cell envelope biogenesis in *Escherichia coli*. *Mol Microbiol* 45:1289–1302.
24. Chng SS, Ruiz N, Chimalakonda G, Silhavy TJ, Kahne D (2010) Characterization of the two-protein complex in *Escherichia coli* responsible for lipopolysaccharide assembly at the outer membrane. *Proc Natl Acad Sci USA* 107:5363–5368.
25. Wu T, et al. (2006) Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci USA* 103:11754–11759.
26. Ruiz N, Kahne D, Silhavy TJ (2009) Transport of lipopolysaccharide across the cell envelope: The long road of discovery. *Nat Rev Microbiol* 7:677–683.
27. Sperandio P, Dehò G, Polissi A (2009) The lipopolysaccharide transport system of Gram-negative bacteria. *Biochim Biophys Acta* 1791:594–602.
28. Kamio Y, Nikaïdo H (1976) Outer membrane of *Salmonella typhimurium*: Accessibility of phospholipid head groups to phospholipase c and cyanogen bromide activated dextran in the external medium. *Biochemistry* 15:2561–2570.
29. Leive L (1974) The barrier function of the gram-negative envelope. *Ann N Y Acad Sci* 235:109–129.
30. Kadokura H, Tian H, Zander T, Bardwell JC, Beckwith J (2004) Snapshots of DsbA in action: Detection of proteins in the process of oxidative folding. *Science* 303:534–537.
31. Tokuda H, Matsuyama S (2004) Sorting of lipoproteins to the outer membrane in *E. coli*. *Biochim Biophys Acta* 1694:1N1–1N9.
32. Endriss F, Braun M, Killmann H, Braun V (2003) Mutant analysis of the *Escherichia coli* FhuA protein reveals sites of FhuA activity. *J Bacteriol* 185:4683–4692.
33. Spiess C, et al. (1997) Biochemical characterization and mass spectrometric disulfide bond mapping of periplasmic alpha-amylase MaS of *Escherichia coli*. *J Biol Chem* 272:22125–22133.
34. Vertommen D, Ruiz N, Leverrier P, Silhavy TJ, Collet JF (2009) Characterization of the role of the *Escherichia coli* periplasmic chaperone SurA using differential proteomics. *Proteomics* 9:2432–2443.
35. Simpson RJ (2008) Estimation of free thiols and disulfide bonds using Ellman's reagent. *Cold Spring Harb Protoc*, 10.1101/pdb.prot4699.
36. Sone M, Kishigami S, Yoshihisa T, Ito K (1997) Roles of disulfide bonds in bacterial alkaline phosphatase. *J Biol Chem* 272:6174–6178.
37. Eser M, Masip L, Kadokura H, Georgiou G, Beckwith J (2009) Disulfide bond formation by exported glutaredoxin indicates glutathione's presence in the *E. coli* periplasm. *Proc Natl Acad Sci USA* 106:1572–1577.
38. Sperandio P, et al. (2008) Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *J Bacteriol* 190:4460–4469.
39. Ruiz N, Gronenberg LS, Kahne D, Silhavy TJ (2008) Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci USA* 105:5537–5542.
40. Chng SS, Gronenberg LS, Kahne D (2010) Proteins required for lipopolysaccharide assembly in *Escherichia coli* form a transenvelope complex. *Biochemistry* 49:4565–4567.