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Use of Single-Cysteine Mutants to Probe the Location of the Disulfide Bond in LamB Protein from Escherichia coli

Richard Ling and Mary Luckey*

Department of Chemistry and Biochemistry
San Francisco State University
San Francisco, CA 94132

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The two cysteine residues of the LamB protein of Escherichia coli outer membrane have been shown to form an intrasubunit disulfide whose location differs greatly in the two current topology models of the LamB protein. This study probes the location of the disulfide by examining conditions for intersubunit disulfide formation in single-cysteine mutants of LamB protein. Formation of an intersubunit bond in the purified mutant proteins, which resulted in a disulfide-linked dimer, only occurred after heat treatment, suggesting the disulfide is not exposed on the surface in the native protein.

The LamB protein in the outer membrane of Escherichia coli functions as part of the maltose transport system, forming a passive transport channel that is selective for maltose and maltodextrins, and as the receptor for bacteriophage lambda and other phages [for reviews see (1) and (2)]. In spite of its lack of extensive sequence homology (3), LamB protein shares many properties with the other outer membrane pore proteins, OmpF, OmpC and PhoE [reviewed in (4)]. It is a homotrimer that most probably forms β barrels transverse to the membrane (5,6). It is highly resistant to heat, detergents and proteolysis (7).

Models for the transmembrane topology of the LamB monomer have been based on the sites of numerous point mutations in the gene which give rise to phage resistance (8) or which affect antibody (9) or starch binding (10). Recently an alternative model was based on prediction of amphiphilic β strands and positioning

*Abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tricine, Tris(hydroxymethyl)-methyl glycine; βME, β-mercaptoethanol; and CNBr, cyanogen bromide.

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aromatic residues at the edge of the hydrophobic core by assigning them arbitrarily high hydrophobicity values (11). Such a hydrophobic girdle has been observed in the crystal structure of OmpF and PhoE (12). One difference between the two models which is amenable to experimental analysis is the environment around the only two cysteines, residues 22 and 38, which form an intrasubunit disulfide bond (13). The original model places the disulfide bond at about the middle of the bilayer, linking the first and second β strands, while the new model places it at the base of the first extracellular loop (from the N terminal), outside the bilayer spanning region.

Chemical modification studies of the cysteines suggested that they were inaccessible in native LamB protein (13). Reaction of the cysteinyl residues with N-ethylmaleimide was only achieved when the LamB trimer (reduced with β-mercaptoethanol) was fully dissociated in 9.4 M urea. No N-ethylmaleimide was incorporated into LamB protein in 4.5 M guanidine with or without reducing agent (14).

The accessibility of the cysteine residues can also be studied in mutant LamB proteins containing only one cysteine per monomer. These mutant proteins, C22S and C38S, were previously reported to form trimers with normal transport properties but reduced thermal stability (13). The present study examines their ability to form intersubunit disulfide bonds.

METHODS

Site-directed mutagenesis and purification of the mutant proteins have been described (13). SDS-PAGE in both one and two dimensions was carried out according to Lugtenberg (15), except when Tricine was used to improve resolution of low molecular weight species (16). Cleavage of purified mutant LamB proteins was obtained with a 5000-fold excess of CNBr in 70% formic acid overnight, conditions previously found to give good cleavage of wild type LamB protein (13).

RESULTS AND DISCUSSION

To investigate the accessibility of the sulfhydryl groups in single-cysteine mutants of LamB protein, their ability to form intersubunit disulfide bonds was examined. When heated in the absence of SDS or reducing agents, the purified C22S and C38S mutant proteins form several discrete higher molecular weight species, in addition to the monomer of 47 kDa and the trimer, whose

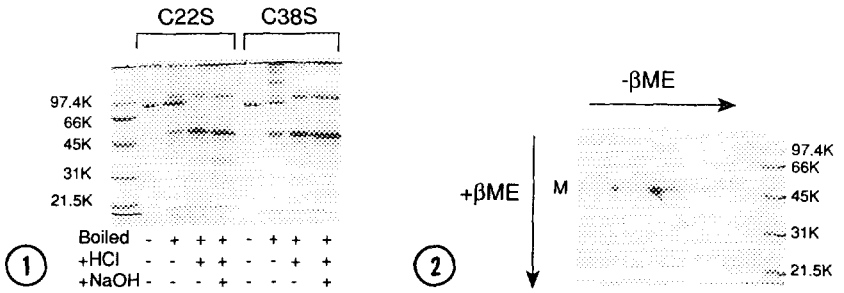
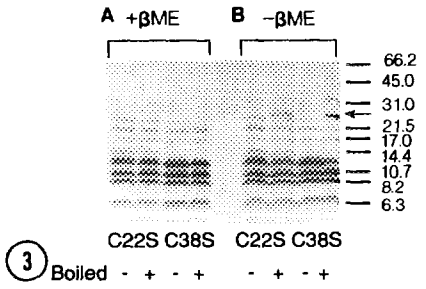


Figure 1. Acid stability of oligomers of C22S and C38S LamB protein.
Mutant proteins were purified by extraction with 2% Triton X-100 in 10 mM EDTA and chromatographed on immobilized maltose binding protein as described previously. Purified proteins were subject to heat treatment as follows: proteins in 10 mM Tris-Cl, pH 7.4, containing 0.02% NaN_3 without SDS or βME were boiled for 10 min, cooled to room temperature and incubated in gel solubilization buffer without βME. SDS PAGE was carried out according to (15). The first lane of each panel shows the untreated proteins, appearing as trimers on the gel. The second lane shows the effect of heat treatment. The boiled proteins were treated with 0.01N HCl (Lane 3 of each panel) and then neutralized with a stoichiometric amount of NaOH (lane 4 of each panel.)

Figure 2. Conversion of the acid-resistant C38S dimer to LamB monomer by reduction.
A sample containing 13 μg of purified C38S LamB protein was treated with 0.01 N HCl after heat treatment for 10 min. SDS PAGE was carried out in two dimensions, with no reducing agent in the first dimension (resulting in both 100 and 47 kDa bands as in Fig.1) and βME added before the second dimension. The mobility of the 100 kDa species was increased to that of the 47 kDa monomer in the second dimension.

abnormal migration corresponds to a mass of 94 kDa (Figure 1). These higher molecular weight species are more pronounced in the C38S protein and absent in the mutant lacking both cysteines (13). Treatment with HCl converted the majority of the high molecular weight species to the species with apparent mass of ca. 100 kDa. Reneutralisation of the solutions did not promote reassociation of the 100 kDa species to trimer, as seen with the wild type monomer (13). The size of the 100 kDa species is approximately that of a denatured dimer if it exhibited normal migration through the SDS-polyacrylamide gel. This dimeric species can be converted to LamB monomer by reduction with βME, indicating it contains a disulfide bond (Figure 2).

To identify the location of the disulfide formed in the single cysteine mutant proteins, the purified proteins with and without heat treatment were digested with CNBr and then analyzed by SDS PAGE in Tricine (16). A new fragment with a mobility corresponding to a mass of 26 kDa appears only when the mutant proteins were subject to the heat treatment before CNBr treatment (Figure 3).



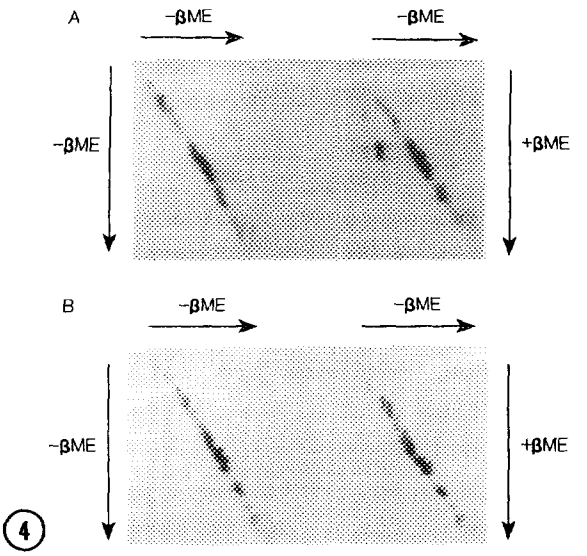


Figure 3. CNBr fragments of C22S and C38S LamB Proteins with and without Heat Treatment.
Samples containing 20 μg of purified C22S and C38S proteins, with and without heat treatment, were treated with a 5000-fold excess of CNBr in 70% formic acid overnight, lyophilized and dissolved in distilled water and gel solubilization buffer with and without βME. After boiling for 5 min the samples were analyzed by SDS-PAGE in tricine buffer. In Panel A, the samples are in 0.3 M βME, while in panel B they do not contain reducing agent. The new fragment with a mobility corresponding to a mass of 26 kDa is present only in samples that were subjected to heat treatment prior to cleavage and electrophoresed in the absence of βME.

Figure 4. Effect of Reducing Agent on the Mobility of the CNBr Fragments in Two Dimensional SDS PAGE.
Two dimensional SDS PAGE in Tricine buffer was carried out with the CNBr fragments shown in Figure 3. Panel A shows fragments of C38S protein after heat treatment electrophoresed with and without βME in the second dimension. Without reducing agent all the fragments lie on the diagonal. When reducing agent is added, one fragment produces a spot lying off the diagonal, indicating its mobility was changed when its disulfide was reduced. In Panel B, fragments of C22S protein which had not been boiled were subject to the same procedure and their mobility was unchanged by reducing agent. Unboiled C38S behaved as seen in B, while boiled C22S behaved as seen in A.

Since the N-terminal fragment containing the cysteinyl residues is 13 kDa (13), the new fragment is formed by an intersubunit disulfide bond linking two N-terminal fragments.

Two dimensional SDS PAGE, with reducing agent introduced after the first dimension, was used to show that the new CNBr fragment is linked by a disulfide. Figure 4A shows the fragments of C38S protein which had been subjected to heat treatment. Addition of βME before running the second dimension increased the mobility of one CNBr fragment, producing a peptide that runs off the diagonal. (The same result is obtained with C22S protein.) When either

mutant protein was not heated prior to CNBr digestion, the mobility of all fragments was the same in both dimensions (Figure 4B), indicating none were linked by a disulfide bond. The intersubunit disulfide bond forms only between two mutant polypeptides after heat treatment.

These results indicate that when each monomer of LamB protein contains a single cysteine, an intersubunit disulfide bond is formed only after perturbing the protein structure by heating. The absence of such a bond in unheated proteins argues that the cysteine residues are not accessible on the surface to form a disulfide bond in the native protein. In contrast, when a purified mutant LamB protein containing a third cysteine in the phage binding site, G382C (17), is concentrated for crystallization, it forms a dimer of trimers, observed with both SDS-PAGE and analytical ultracentrifugation (18). The dimer of trimers could be partially dissociated to trimers by treatment with dithiothreitol. Thus in this case a surface-exposed cysteine can link the native trimers by an intersubunit disulfide bond.

While the lack of intersubunit disulfide bond formation in unheated C22S and C38S proteins suggests that these cysteine residues are not exposed on the surface like G382C, it is possible that spatial restrictions prevent bond formation. Further biochemical studies will address the nature of the environment around the cysteine residues in wild type LamB protein.

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