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A chemically cross-linked nonlinear proOmpA molecule can be translocated into everted membrane vesicles of *Escherichia coli* in the presence of the proton motive force

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The chemical cross-linking between the two cysteine residues at positions +290 and +302 of proOmpA was performed with *N,N'*-bis(3-maleimido-propionyl)-2-hydroxy-1,3-propanediamine. In the absence of the proton motive force ($\Delta\tilde{\mu}H^+$), the cross-linked proOmpA was only partially translocated into everted membrane vesicles, leading to accumulation of translocation intermediates. In the presence of $\Delta\tilde{\mu}H^+$, the cross-linked proOmpA was completely translocated. The translocated OmpA still possessed the cross-linked loop composed of 13 amino acid residues and the cross-linker. It is concluded that polypeptide chains need not be necessarily linear and fully expanded to be translocated.

Cross-linking; Everted membrane vesicle; ProOmpA; Protein secretion; Proton motive force

1. INTRODUCTION

The mechanisms underlying protein translocation across biological membranes have been the subject of extensive studies. It has been suggested that secretory proteins are translocated as an expanded linear polypeptide chain through a channel in the secretory machinery [1–3]. In a previous work we found, however, that proOmpA, the precursor of an outer membrane protein of *Escherichia coli*, can be translocated in vitro into everted membrane vesicles of the cytoplasmic membrane in the presence of the proton motive force ($\Delta\tilde{\mu}H^+$), even when the protein possesses a disulfide bridge between the cysteine residues [4]. This suggests that a nonlinear polypeptide chain can be accepted by the secretory machinery. Since the formation of a disulfide bridge is a reversible reaction, there is the possibility that the disulfide bridge underwent cleavage and reformation during the translocation reaction, however.

In the present work, therefore, cross-linking between the two cysteine residues was performed using an uncleavable cross-linker. The cross-linked proOmpA possessing a loop comprising 13 amino acid residues and the cross-linker was translocated into everted membrane vesicles when $\Delta\tilde{\mu}H^+$ was imposed.

Abbreviations: MPHP, *N,N'*-bis(3-maleimide-propionyl)-2-hydroxy-1,3-propanediamine; SDS, sodium dodecylsulfate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E. coli K003 (Lpp$^-$ *ΔuncB-C*-Tn10) [5] was used. Plasmid pSI053 [6] contains the *ompA* gene that codes for proOmpA. Plasmid pKT001 [4] contains the mutant *ompA* gene that codes for a proOmpA in which Cys-302 is replaced with Gly.

2.2. *Materials*

Tran^{35}S-label, a mixture of 70% [^{35}S]methionine and 20% [^{35}S]cysteine, 1000 Ci/mmol, was obtained from ICN. L-[^{35}S]cysteine, approximately 1000 Ci/mmol, was obtained from Amersham. *N,N'*-bis(3-maleimido-propionyl)-2-hydroxy-1,3-propanediamine (MPHP) was from Sigma and dissolved in dimethyl sulfoxide. Lysyl endopeptidase was from Wako Pure Chemical Industries. Proteinase K was from Merck. The SecA protein was purified as described [7]. The protein molecular weight markers were from BRL.

2.3. *Preparation of everted membrane vesicles*

Everted membrane vesicles were prepared from *E. coli* K003 (*ΔuncB-C*) cells as described previously [6]. The amount of membrane vesicles was expressed as that of protein, which was determined by the method of Lowry et al. [8].

2.4. *Transcription and translation reactions and removal by gel filtration of small molecules from the reaction mixture after translation*

In vitro transcription and translation reactions were carried out as described [9,10]. The reaction mixture was then gel-filtered through a Sephadex G-75 column as described [10].

2.5. *Cross-linking reaction*

The gel-filtered fraction (50 μl) containing ^{35}S-labeled proOmpA was mixed with 47 μl of 50 mM potassium phosphate (pH 7.5). 3 μl of 16.7 mM MPHP was then added and the cross-linking reaction was carried out at room temperature.

2.6. *Lysyl endopeptidase digestion of [^{35}S]cysteine-labeled proOmpA*

Cross-linking of [^{35}S]cysteine-labeled proOmpA was carried out for 30 min as described above. Dithiothreitol (DTT) was then added

to the cross-linked ^{35}S-labeled proOmpA preparation to a final concentration of 4 mM. 20 μl of it was then mixed with 25 μl of 200 mM Tris-HCl (pH 8.8) containing 5 μg of bovine serum albumin. 5 μl of 0.7 mg/ml lysyl endopeptidase was then added and the digestion was carried out at 30°C for 1.5 h.

2.7. *In vitro translocation of cross-linked proOmpA*

The cross-linking of proOmpA was carried out for 1 h as described above. The sample was then treated with 10 mM DTT. Trichloroacetic acid was then added to the mixture to a final concentration of 10%, and the resulting precipitate was successively washed with acetone and ethyl ether, dried and dissolved in 40 μl of 8 M urea, 50 mM Tris-HCl (pH 8.0), 20 mM DTT. For the translocation reaction, 6 μl of this solution and 49 liters of 50 mM potassium phosphate (pH 7.5), 7 mM $MgSO_4$, 5 mM DTT, containing 0.18 μg of purified SecA and 10 μg of everted membrane vesicles, were mixed. The translocation reaction was carried out at 37°C for 12 min after the addition of 5 μl of 60 mM ATP, 60 mM NADH. The reaction mixture was then treated with 5 μl of 5 mg/ml proteinase K for 20 min on ice and the translocated protein, which was proteinase K-resistant, was detected by SDS-polyacrylamide gel electrophoresis (PAGE) by means of fluorography as described [4].

2.8. *Treatment of proOmpA with ferricyanide*

^{35}S-Labeled proOmpA possessing a disulfide bridge was prepared in the presence of ferricyanide as described [4] and then dissolved in 8 M urea, 50 mM Tris-HCl (pH 8.0).

3. RESULTS

3.1. *Intramolecular cross-linking of proOmpA between Cys-290 and Cys-302 with MPHP*

To cross-link the two cysteine residues at positions +290 and +302 intramolecularly and irreversibly, [^{35}S]methionine-labeled proOmpA synthesized in vitro was treated with MPHP, a cysteine specific bifunctional cross-linker, and the cross-linked sample was analyzed by SDS-PAGE. A single radioactive band was observed at a position slightly higher than that of untreated proOmpA, suggesting the chemical binding of the cross-linker to proOmpA (Fig. 1A, lanes 1 and 2). Formation of proOmpA multimers due to intermolecular cross-linking was not observed.

We then examined whether the cross-linking between the two cysteine residues occurred intramolecularly or not. ProOmpA possesses 3 lysine residues near the 2 cysteine residues (Lys-273, Lys-294 and Lys-314) [11]. The digestion of native proOmpA with lysyl endopeptidase, which is specific to the carboxyl side of lysine residues [12,13], should, therefore, result in the formation of two cysteine-possessing peptides, Ile-274–Lys-294 and Glu-295–Lys-314 (Fig. 2). A [^{35}S]cysteine-possessing peptide of a predicted size (about 2 kDa) appeared upon digestion of native proOmpA with lysyl endopeptidase (Fig. 1B, lanes 3 and 4). When the MPHP-treated proOmpA was digested similarly, a [^{35}S]cysteine-possessing band corresponding to a molecular mass of about 4 kDa appeared (Fig. 1B, lanes 1 and 2). This was the same size as peptide Ile-274–Lys-314 which is the result of cross-linking between Cys-290 and Cys-302 through MPHP followed by cleavage at Lys-294–Gln-295 (Fig. 2). We conclude, therefore, that

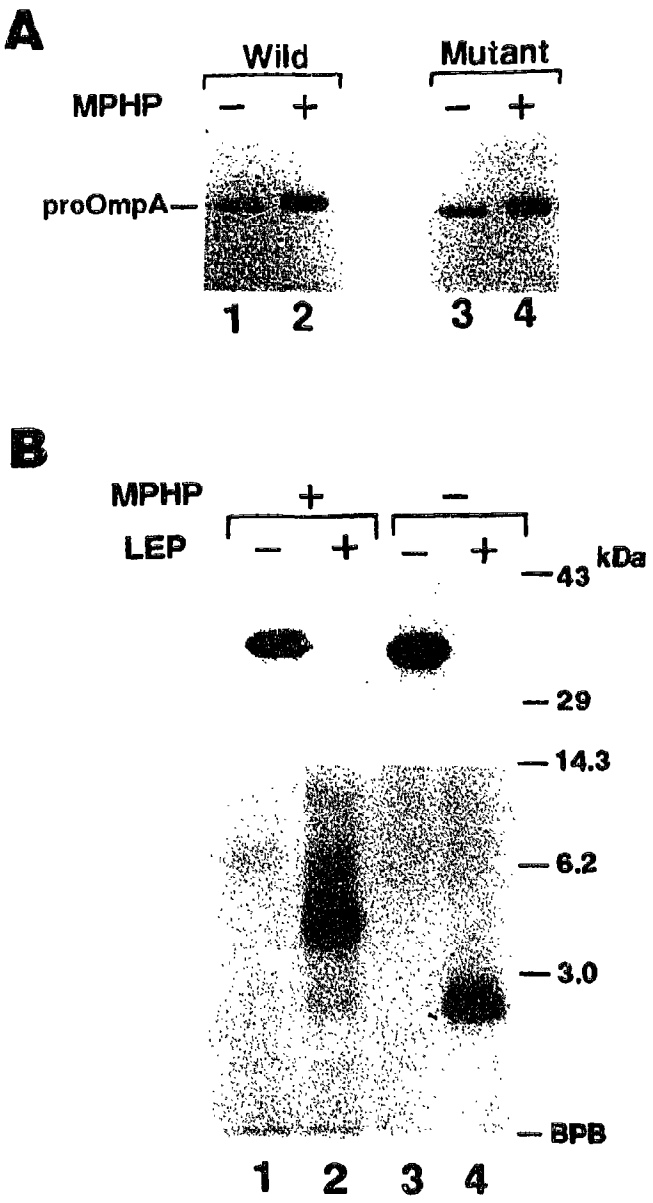

Fig. 1. MPHP-treatment of proOmpA resulted in intramolecular cross-linking between Cys-290 and Cys-302. (A) [^{35}S]Methionine-labeled wild-type proOmpA (lanes 1 and 2) or the mutant proOmpA (lanes 3 and 4), in which Cys-302 is replaced with Gly, was treated with (lanes 2 and 4) or without (lanes 1 and 3) 500 μM MPHP for 1 h. DTT was added to the reaction mixture to a final concentration of 10 mM. Trichloroacetic acid was then added to the mixture. The resulting precipitate was successively washed with acetone and ethyl ether, and analyzed by 14% SDS-PAGE, followed by fluorography. Lanes 2 and 4 contained two-fold amounts of the gel-filtered fractions, respectively, compared with lanes 1 and 3, respectively. Only the relevant regions are shown. The migration position of proOmpA is indicated. (B) [^{35}S]Cysteine-labeled wild-type proOmpA was treated with (lanes 1 and 2) or without (lanes 3 and 4) 500 μM MPHP for 30 min. The reaction was terminated by adding DTT to a final concentration of 4 mM. The samples were then incubated with (lanes 2 and 4) or without (lanes 1 and 3) lysyl endopeptidase (LEP), followed by precipitation with trichloroacetic acid. The resulting precipitate was then analyzed by 15% SDS-PAGE. Only the relevant regions are shown. The migration positions of prestained protein markers and Bromophenol blue dye (BPB) are indicated at the right.

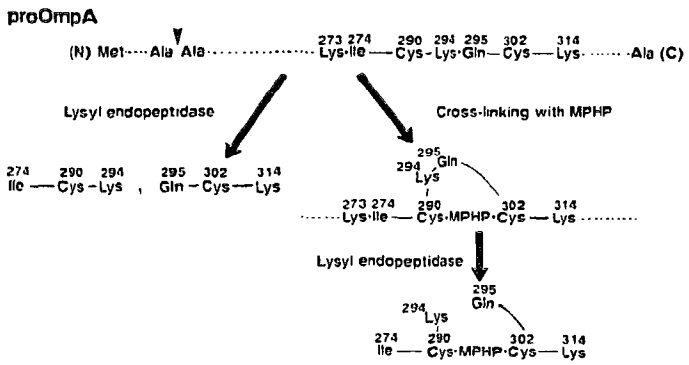

Fig. 2. Chemical structures of polypeptides produced upon lysyl endopeptidase-digestion of proOmpA.

the MPHP-treatment resulted in intramolecular cross-linking between the two cysteine residues. The 2 kDa band after the cross-linking was very weak, indicating that the cross-linking took place almost quantitatively.

3.2. *Cross-linked proOmpA can be translocated into everted membrane vesicles in the presence of* $\Delta\tilde{\mu}H^+$

In a previous work [4], we demonstrated that ferricyanide-oxidized proOmpA possessing an intramolecular disulfide bridge between the two cysteine residues can be completely translocated into everted membrane vesicles in the presence of $\Delta\tilde{\mu}H^+$. In the absence of $\Delta\tilde{\mu}H^+$, on the other hand, the translocation reaction proceeded only partially, leading to the accumulation of translocation intermediates represented by bands A and B on an SDS-gel after proteinase K digestion, indicating that the translocation does not proceed beyond the site where the disulfide bridge is formed [4]. We confirmed these phenomena in the present work (Fig. 3A, lanes 1 and 2). We further demonstrated that the oxidized proOmpA became translocatable, irrespective of the presence or absence of $\Delta\tilde{\mu}H^+$, when it was treated with DTT (Fig. 3A, lanes 3 and 4).

Essentially the same experiment was carried out using proOmpA intramolecularly cross-linked with MPHP (Fig. 3B). The complete translocation of the cross-linked proOmpA was also observed, when $\Delta\tilde{\mu}H^+$ was imposed. In the absence of $\Delta\tilde{\mu}H^+$, on the other hand, the translocation of the cross-linked proOmpA resulted in the appearance of bands at the positions of bands A and B even in the presence of DTT. As far as the positions on an SDS-polyacrylamide gel are concerned, the band A and B materials were the same as those for the disulfide bridge-possessing proOmpA. From these results, we conclude that the cross-linked proOmpA can be completely translocated into everted membrane vesicles in the presence of $\Delta\tilde{\mu}H^+$ and that its translocation stops at the site of cross-linking in the absence of $\Delta\tilde{\mu}H^+$.

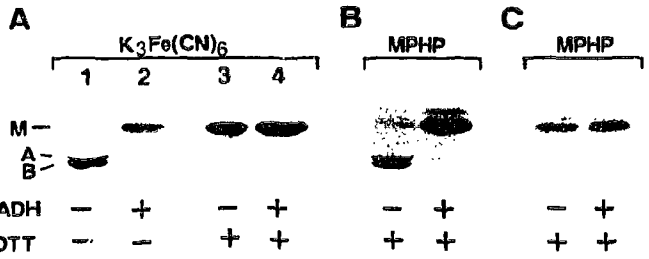

Fig. 3. Effects of cross-linking with MPHP on the translocation of the wild-type and mutant proOmpAs into membrane vesicles. (A) [^{35}S]Methionine-labeled wild-type proOmpA was treated with ferricyanide and then dissolved in 8 M urea, 50 mM Tris-HCl (pH 8.0) (lanes 1 and 2) or 8 M urea, 50 mM Tris-HCl (pH 8.0), 20 mM DTT (lanes 3 and 4). (B) [^{35}S]Methionine-labeled wild-type proOmA was treated with 500 M MPHP for 1 h and then dissolved in 8 M urea, 50 mM Tris-HCl (pH 8.0), 20 mM DTT. (C) [^{35}S]Methionine-labeled mutant proOmpA, in which Cys-302 is replaced with Gly, was treated with 500 M MPHP as in (B). (A-C) The proOmpA samples thus treated were subjected to the translocation reaction at 37°C for 12 min in the presence or absence of 6 mM DTT and 5 mM NADH as indicated. The concentration of ATP was 5 mM. After the incubation, the reaction was terminated on ice. The translocation mixture was then treated with proteinase K and analyzed by 12.5% SDS-PAGE, followed by fluorography. The positions of mature OmpA (M) and the band A and B materials are indicated.

3.3. *MPHP modification without intramolecular cross-linking does not block translocation*

To exclude the possibility that the modification with MPHP itself prevents the translocation of proOmpA and consequently results in the accumulation of translocation intermediates, an experiment was performed using a mutant proOmpA which possesses only one cysteine residue and hence can be modified by MPHP but cannot be cross-linked intramolecularly. The mutant proOmpA preparation was treated with MPHP and then with DTT to inactivate unreacted maleimide groups. The resultant specimen gave a single band on an SDS-gel which migrated slightly slower than that of the nontreated one (Fig. 1A, lanes 3 and 4), suggesting that the cysteine residue of the mutant proOmpA had been modified by the cross-linker. Since the MPHP-treated mutant proOmpA had been treated with DTT, another maleimide group of this bifunctional cross-linker should have reacted with the latter molecule. Differing from the MPHP-treated wild-type proOmpA, the MPHP-treated mutant proOmpA was translocated completely in both the presence and absence of $\Delta\tilde{\mu}H^+$ (Fic. 3C). It is likely, therefore, that the introduction of an MPHP molecule, and probably two of them, into proOmpA does not change the $\Delta\tilde{\mu}H^+$ requirement for the translocation.

3.4. *The translocated OmpA still possesses the cross-linked polypeptide loop*

As shown by the experimental results in Fig. 4, we directly demonstrated that the MPHP-modified pro-

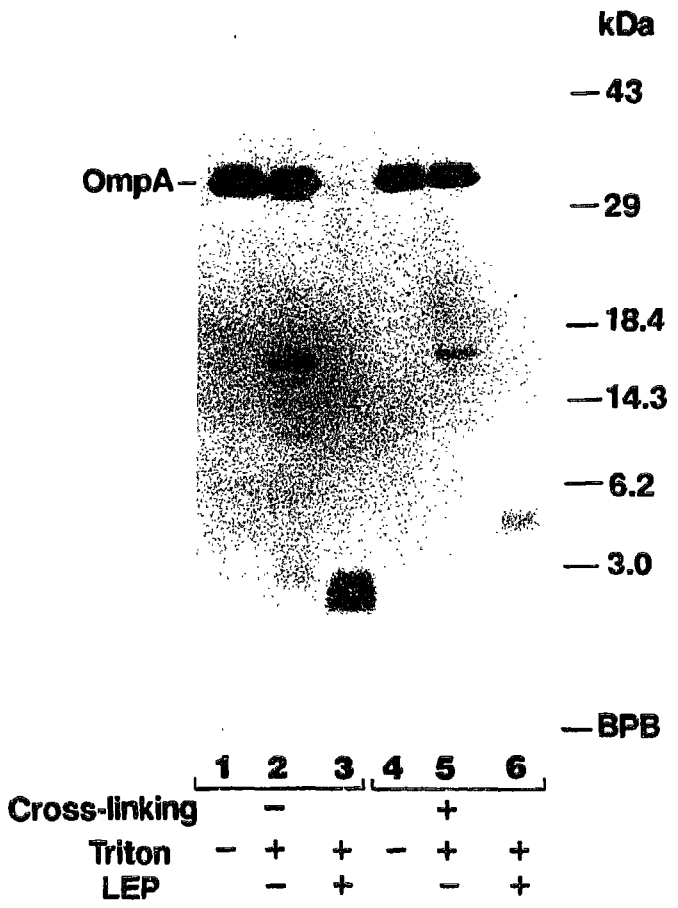

Fig. 4. Lysyl endopeptidase-digestion profiles of uncross-linked and cross-linked OmpA after translocation. [^{35}S]Cysteine-labeled wild-type proOmpA was treated with (lanes 4-6) or without (lanes 1-3) MPHP, and then the translocation reaction was carried out in the presence of 5 mM ATP, 5 mM NADH and 6 mM DTT as described in the legend to Fig. 3. After proteinase K-treatment, 135 μl of 6 M urea, 50 mM potassium phosphate (pH 7.5), 1 mM phenylmethylsulfonyl fluoride was added to the reaction mixture (65 μl). The samples were then centrifuged at 50 000 rpm for 30 min at 7°C to recover membrane vesicles (lanes 1 and 4). The membrane vesicles were then dissolved in 45 μl of 0.11% Triton X-100, 55 mM Tris-HCl (pH 8.8) and treated with 5 μl of H_2O (lanes 2 and 5) or 0.7 mg/ml lysyl endopeptidase (LEP) (lanes 3 and 6) for 1 h at 30°C. All samples were analyzed by 15% SDS-PAGE. The migration positions of OmpA, prestained protein markers and Bromophenol blue dye (BPB) are indicated.

OmpA, which was translocated into everted membrane vesicles in the presence of $\Delta\tilde{\mu}H^+$, still possessed the loop composed of 13 amino acid residues and MPHP (see Fig. 2). [^{35}S]Cysteine-labeled proOmpA was synthesized, cross-linked with MPHP and then subjected to the translocation reaction in the presence of NADH. After proteinase K-treatment, membrane vesicles were recovered, treated with lysyl endopeptidase in the presence of Triton X-100 and then analysed on an SDS-gel.

The MPHP-treated and translocated OmpA, which appeared to be a proteinase K-resistant mature form, gave rise to a cysteine-possessing 4 kDa band material upon digestion with lysyl endopeptidase (Fig. 4, lane 6), as in the case of the MPHP-treated proOmpA used as the substrate (Fig. 1B, lane 2). This material was not formed in the absence of lysyl endopeptidase (Fig. 4, lane 5). When proOmpA, which had not been treated with MPHP, was used, the lysyl endopeptidase-treatment of the translocated protein resulted in the appearance of a 2 kDa band material (Fig. 4, lane 3), which corresponds to the cysteine-containing fragments, Ile-273-Lys-294 and Gln-295-Lys314 (see Figs. 2 and 1B). Taking all these results together, we conclude that proOmpA possessing the loop comprising 13 amino acid residues and MPHP can be translocated into everted membrane vesicles as it is, when the vesicles are energized.

4. DISCUSSION

In the present work, we demonstrated that proOmpA chemically cross-linked between the two cysteine residues with MPHP was translocated into energized membrane vesicles. Energization was essential for the translocation of this compound. Since the chemical cross-linking was an irreversible process and since the translocated compound still possessed the original loop structure, we conclude that the secretory machinery tolerates the translocation of a protein possessing such a loop structure; i.e. polypeptide chains do not necessarily have to be linear and fully expanded.

The cross-linked loop is composed of 13 amino acid residues and MPHP, the total molecular mass being 1841 Da. The translocation of polypeptide chains through a pore in the secretory machinery has been considered. The passage of such a cross-linked loop would not support the presence of such a pore, or a closed channel, as a path for secretory proteins. It is possible that polypeptide chains are recognized by an open channel or the surface of the secretory machinery.

Another interesting question which arose in the present work is what the principal structure that is recognized by the secretory machinery is. The passage of proOmpA possessing the cross-linked loop still allows us to contend that the backbone of polypeptide chains is the target of such recognition and that the polypeptide chain is translocated along the backbone chain. Alternatively, it may be possible that the translocation reaction advances through the cross-linker, recognizing the polypeptide loop as a large side chain. In the latter case, the substance to be translocated through the secretory machinery need not be necessarily solely held together by polypeptide bonds.

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