Preprotein Translocation Creates a Halide Anion Permeability in the Escherichia coli Plasma Membrane*

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The electrochemical potential drives the translocation of the precursor form of outer membrane protein A (proOmpA) and other proteins across the plasma membrane of Escherichia coli. We have measured the electrical potential, \( \Delta \psi \), across inverted membrane vesicles during proOmpA translocation. \( \Delta \psi \), generated by the electron transport chain, is substantially dissipated by proOmpA translocation. \( \Delta \psi \) dissipation requires SecA, ATP, and proOmpA which, due to the covalent addition of a folded protein to a cysteinyll side chain, is arrested during its translocation, can nevertheless cause the loss of \( \Delta \psi \). Thus the movement of charged amino acyl residues is not dissipating the potential. This translocation-specific reduction in \( \Delta \psi \) is only seen in the presence of halide anions, although halide anions are not needed for proOmpA translocation per se. We therefore propose that translocation intermediates directly increase the membrane permeability to halide anions.

Through the convergence of genetic and enzymological studies, the major elements of protein translocation across the E. coli plasma membrane have now been described (Wickner et al., 1991; Bieker et al., 1990; Schatz and Beckwith, 1990), making this the premier system for mechanistic studies of translocation. Preproteins are made with NH\(_2\)-terminal leader sequences. In the cytosol, preproteins such as proOmpA (the precursor form of outer membrane protein A) may form a stoichiometric complex with SecB (Lecker et al., 1989, 1990; Kumamoto, 1989), preventing aggregation and contributing to targeting specificity (Hartl et al., 1990). Transfer across the plasma membrane is catalyzed by preprotein translocase, a multisubunit enzyme (Brundage et al., 1990). Translocon consists of a peripheral membrane domain, the SecA protein, which is bound to the membrane via its affinities for acidic phospholipids (Lill et al., 1990; Hendrick and Wickner, 1991) and for the SecY/E integral membrane protein. The latter is comprised of three components, the SecY and SecE polypeptides and one polypeptide of as yet unidentified gene or function (Brundage et al., 1990). The proOmpA - SecB complex binds to translocase at SecA by virtue of the affinities of SecA for SecB (Hartl et al., 1990) and for the leader and mature domains of the preprotein (Lill et al., 1990). ATP binding then causes an initial transfer of a loop of the protein across the membrane (Schiebel et al., 1991). Upon nucleotide hydrolysis, proOmpA is released from tight SecA association and can undergo a rapid completion of translocation, driven by both \( \Delta \psi \) and \( \Delta p \)H (Schiebel et al., 1991).

Despite extensive study, the role of \( \Delta \psi \) and \( \Delta p \)H in preprotein translocation has remained unclear. In vitro studies showed the following: 1) the export of virtually all proteins, including the translocase-independent M13 procot, requires the membrane potential (DATE et al., 1986; Daniels et al., 1981; Zimmermann and Wickner, 1983). 2) Each protein requires a different level of potential for its export (Daniels et al., 1981), and mutations can strongly affect the response of a protein to a potential (Zimmermann et al., 1982). 3) Translocation can be supported by either \( \Delta \psi \) or \( \Delta p \)H (Bakker and Randall, 1984). In vitro translocation is not as strictly dependent on the membrane potential. However, translocation driven by ATP alone is strikingly inefficient in that approximately 5,000 ATP molecules are consumed to translocate one molecule of proOmpA. Optimal translocation rates and efficiencies require both ATP and the membrane potential both in crude in vitro reactions (Geller et al., 1986) and in translocation as reconstituted with purified components (Brundage et al., 1990).

It has recently been reported that E. coli plasma membranes contain channels of large conductance (Simon et al., 1989), although the relation of these channels to preprotein transport is not yet established. Channels which might be involved in protein translocation have also been described in the endoplasmic reticulum (Simon and Blobel, 1991). We have sought to exploit the availability of large amounts of pure proOmpA (Crooke et al., 1988) to determine whether the translocation of preprotein, as it moves, may bear net charge across the membrane and thereby "consume" either component of the electrochemical potential. Rather than finding such dissipation of potential by a transiting polypeptide chain, we now report that translocation intermediates, even when arrested in their motion, cause an increased membrane permeability to halide anions.

MATERIALS AND METHODS

Inverted inner membrane vesicles were prepared from E. coli strain KM9 (Cunningham et al., 1989) as described (Chang et al., 1978).
They were equilibrated by sonication with buffer A (50 mM Tris-SO₄, pH 7.5, 25 mM KCl, 10 mM MgSO₄) and stored in this buffer. The following proteins were purified according to published methods: SecA protein (Cunningham et al., 1989), SecB (Weiss et al., 1988), as well as SecA-Lecker (Lecker et al., 1989), proOmpA (Croke and Wickner, 1987), unlabeled proOmpA (Croke et al., 1988), proOmpA-SPDP-BPTI (Schiebel et al., 1991), and [²⁵³⁴S]ethylmaleimide-proOmpA (Schiebel et al., 1991). proOmpA leader peptide was synthesized chemically and purified using high pressure liquid chromatography (Cunningham and Wickner, 1988). [²⁰³⁵C]Methylamine hydrochloride (2.00 GBq/mmol) and [³⁵Cl]thiocyanate (2.07 GBq/mmol) were from Amersham Corp. Creatine kinase, NADH, and proteinase K were from Boehringer Mannheim.

**RESULTS**

While there have been many studies of the effects of a membrane electrochemical potential on preprotein translocation, the effects of translocation on the establishment and maintenance of a potential have not been examined. To study these effects, we used inverted inner membrane vesicles of *E. coli* KM9. Since this *E. coli* strain does not have a functional *F*₁/F₀ ATPase (Cunningham et al., 1989), an electrochemical potential can only be induced by the electron transport chain. proOmpA cross-linked to bovine pancreas tryptic inhibitor (proOmpA-BPTI) was used to saturate the translocation sites (Schiebel et al., 1991). Translocation of proOmpA-BPTI results in a translocation intermediate where the amino-terminal 80% of proOmpA has been translocated.

**Electrical Potential Dissipation by Translocating proOmpA**—The electrical potential of membrane vesicles induced by the electron donors NADH or succinate was measured with the fluorescent dye oxonol VI (Fig. 1). In the absence of translocation intermediate, a decrease of the relative fluorescence was observed after the addition of NADH to membranes (Fig. 1A). The maximum decrease of the fluorescent signal was taken as the electrical potential. The starting level of fluorescence was restored after the electrical potential was dissipated by the drug valinomycin. The NADH-induced electrical potential of membranes bearing the translocation intermediate (Fig. 1B) was significantly lower than that of the control membranes. A similar result was obtained with succinate as the electron donor (data not shown).

To exclude the possibility that the translocation intermediate directly inhibits the electron transport chain, we measured the oxidation of NADH. The NADH oxidative activity of membranes was unaffected by the translocation intermediate (data not shown). In addition, as shown below, the electrical potential of both membrane populations were the same under other buffer conditions (see Fig. 6 and Table I). The translocation intermediate does not affect the activity of the electron transport chain, but rather the translocation intermediates dissipate the electrical potential induced by the electron transport chain.

An electrochemical potential might be uncoupled by leader peptides. We therefore tested whether the electrical potential was dissipated by the leader peptide of proOmpA. Inner membrane vesicles were incubated with increasing concentrations of leader peptide, and the electrical potential was measured using oxonol VI. The relative fluorescence of oxonol VI responded linearly to the amount of inner membrane vesicles added, indicating that the measurements were performed in the linear range of the assay (data not shown). Leader peptide up to 25 μM had only a moderate effect on the electrical potential (Fig. 2). Membranes incubated with 25 μM [¹⁴C]labeled leader peptide were sedimented through a sucrose solution to estimate the amount of leader peptide bound to the membranes. Seventy-five percent of the peptide sedimented with the vesicles, while no peptide was recovered in the pellet when membranes were omitted. The proOmpA-BPTI concentration used to saturated the translocation sites was 67 nm, far below the concentrations of leader peptide needed to affect

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**FIG. 1.** The Δψ of membranes bearing the translocation intermediate (B) is lower than that of membranes not bearing the intermediate (A). Membrane vesicles, SecA, SecB, and ATP were incubated with (B) or without (A) proOmpA-SPDP-BPTI as described under "Materials and Methods." The vesicles were centrifuged (100,000 × g, 1 h, 2 °C) and the sediment resuspended in an equal volume of buffer A, 2 mM ATP, 0.2 mg/ml BSA. Vesicles were bath-sonicated for 10 s and equilibrated with the buffer for 1 h on ice. Oxonol VI (2 μM) was added, and the fluorescence was measured at 25 °C. NADH (5 mM) was added as indicated, followed by valinomycin (1 μM).
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Table I

| Anion   | Cation     | Control membranes | Membranes bearing translocation intermediate |
|---------|------------|-------------------|-------------------------------------------|
| Sulfate | Potassium  | 100               | 99                                        |
| Fluoride| Sodium     | 93                | 85                                        |
| Chloride| Potassium  | 84                | 65                                        |
| Bromide | Potassium  | 64                | 44                                        |
| Iodine  | Potassium  | 23                | 16                                        |
| Sulfate | Tris       | 100               | 108                                       |
| Sulfate | Lithium    | 99                | 102                                       |
| Sulfate | Sodium     | 99                | 102                                       |
| Sulfate | Potassium  | 100               | 102                                       |

Effect of ions on translocation intermediate induced dissipation of $\Delta \psi$

Membrane vesicles (with or without the translocation intermediate) were prepared as described in Fig. 7. Sedimented membrane vesicles were resuspended in an equal volume of 50 mM Tris-SO$_4$, pH 7.5, 10 mM MgSO$_4$, 2 mM ATP, 0.2 mg/ml BSA. Samples were divided into aliquots, and 5 mM of NaF, KCl, KBr, KI, 3.3 mM of K$_2$SO$_4$, or 12.5 mM of Tris-SO$_4$, pH 7.5, Li$_2$SO$_4$, Na$_2$SO$_4$, or K$_2$SO$_4$ was added. The samples were bath sonicated (10 s) and incubated for 1 h on ice. $\Delta \psi$ was measured by oxonol VI fluorescence with NADH as electron donor. Fluorescence is expressed as percent of signal obtained using control membranes in K$_2$SO$_4$ or Tris-sulfate-containing buffer.

The samples were bath sonicated (10 s) and incubated for 1 h on ice. $\Delta \psi$ was measured by oxonol VI fluorescence with NADH as electron donor. Fluorescence is expressed as percent of signal obtained using control membranes in K$_2$SO$_4$ or Tris-sulfate-containing buffer.

![Graph](image)

**FIG. 2.** ProOmpA leader peptide does not affect $\Delta \psi$. Membrane vesicles (100 µg/ml) in buffer A and 0.2 mg/ml BSA were incubated with 0, 0.5, 2.5, 5.0, 10, or 25 µM proOmpA leader peptide. The samples were incubated for 10 min at 37°C. $\Delta \psi$ was measured by oxonol VI fluorescence in response to addition of NADH. Fluorescence is expressed as percent of the control, maximal signal, which was assayed by flow dialysis to be approximately 100 mV.

![Graph](image)

**FIG. 3.** Dissipation of $\Delta \psi$ is dependent on translocation conditions. KM9 membrane vesicles in buffer A containing SecB, glutathione, and BSA were incubated for 20 min at 37°C with SecA (lane 1), SecA and ATP (lane 2), SecA, ATP, and proOmpA-BPTI (lane 3), SecA, ATP, and proOmpA (lane 4), SecA and proOmpA-BPTI (lane 5), or ATP and proOmpA-BPTI (lane 6). $\Delta \psi$ was measured by oxonol VI fluorescence at 35°C after the addition of NADH. Fluorescence is expressed as percent of maximal signal, which was assayed by flow dialysis to be approximately 100 mV. The average of four measurements is shown. The bars indicate the standard deviation ($n = 4$).

![Graph](image)

**FIG. 4.** Dissipation of $\Delta \psi$ is caused by translocation intermediate. In vitro translocation of proOmpA-SPDP-BPTI (see "Materials and Methods") was performed with 0, 2.5, 7.5, 22.5, 67.5, or 135 nM proOmpA-SPDP-BPTI. Vesicles bearing the translocation intermediate (0.7 ml) were sedimented (100,000 × g, 1 h, 2°C) through a sucrose solution (3.1 ml, 0.2 M sucrose, 50 mM Tris-SO$_4$, pH 7.5, 25 mM KCl, 5 mM MgSO$_4$) to remove non-translocated proOmpA-BPTI. The sediment was resuspended in an equal volume of buffer A containing 2 mM ATP, 0.2 mg/ml BSA, 100 µM glutathione (oxidized). Samples were divided in half and incubated with (open squares) or without (closed squares) 2 mM DTT for 20 min at 37°C in the presence of 40 µg/ml SecA. Six parts of each sample were used to measure $\Delta \psi$ by oxonol VI fluorescence in response to addition of NADH. Fluorescence is expressed as percent of maximal signal. B, to one part, 20 µg/ml SecB and 8 µg/ml $[^{35}S]$-N-ethylmaleimide-proOmpA (500 cpm/ng pro-OmpA) were added and the samples incubated for 10 min at 37°C. N-Ethylmaleimide-modified proOmpA was used because its translocation is independent on the DTT concentration. The translocation reactions were stopped by chilling on ice. Samples were incubated with 0.8 mg/ml proteinase K for 20 min on ice and concentrated by trichloroacetic acid precipitation, and proteins were separated by SDS-PAGE. Gels were analyzed by fluorography and laser scanning.

In these studies, BPTI was cross-linked to proOmpA via the reducible cross-linker SPDP. The BPTI of the translocation intermediate can be released by the reducing agent diethiothreitol (DTT) and the translocation of the proOmpA completed by a further incubation with ATP (Schiebel et al., 1991). We used this property of the translocation intermediate to investigate whether dissipation of the electrical potential

the electrochemical potential. The interaction of the proOmpA leader alone with the membranes cannot be responsible for the dissipation of the electrical potential caused by the translocation intermediate.

Control experiments repeatedly showed that the dissipation of the electrical potential requires translocation (Fig. 3), which is itself SecA- and ATP-dependent. The electrical potential was only diminished upon the addition of SecA, ATP, and proOmpA-BPTI (lane 3). The incubation of the membrane vesicles with only ATP, SecB (data not shown), or proOmpA-BPTI in the absence of ATP (lane 5) did not influence the electrical potential. Some SecA remained bound to the membrane vesicles during the isolation procedure. However, the dissipation of the electrical potential was clearly increased by the addition of SecA to the translocation reaction (compare lane 6 with lane 3), as was translocation. proOmpA translocation without induced translocation arrest also dissipated the electrical potential (lane 4), whereas OmpA had no effect (data not shown).
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can be reversed by completing translocation. Inner membrane vesicles were incubated with increasing concentrations of proOmpA-BPTI. After a 20-min translocation reaction, the vesicles were centrifuged through a sucrose solution to remove untranslocated proOmpA-BPTI. Membranes were resuspended and incubated with or without DTT in the presence of ATP. Where added, DTT allowed release of the BPTI from the proOmpA and completion of proOmpA translocation. The electrical potential was measured after NADH addition (Fig. 4A). A second translocation reaction was performed with [35S] proOmpA as an assay of the number of free translocation sites (Fig. 4B). The dissipation of the electrical potential was clearly dependent on the concentration of added proOmpA-BPTI (Fig. 4A, filled squares). Half-maximal dissipation of the electrical potential was seen at approximately 10 nM proOmpA-BPTI. The number of free translocation sites correlated with the electrical potential (compare Fig. 4, panel A with panel B, filled squares). All the translocation sites were saturated by incubating the membrane vesicles with 67 nM proOmpA-BPTI, a concentration which also maximally dissipated the Δψ. Upon completion of translocation after the addition of DTT, the dissipation of the electrical potential was relieved (Fig. 4A, open squares) and, in parallel, there were an increased number of free translocation sites (Fig. 4B, open squares). We conclude that the dissipation of the electrical potential is caused by the mature part of the membrane-spanning translocation intermediate and not by the leader peptide.

The Translocation Intermediate Does Not Affect ΔH—Proton movement in response to a translocation intermediate should affect both components of the electrochemical potential, ΔH as well as the electrical potential Δψ. Flow dialysis with the membrane permeable cation methylamine was used to measure the ΔH of membranes with or without the translocation intermediate (Fig. 5). The buffer concentration was decreased to 20 mM to allow the measurement of small changes in ΔH; this buffer concentration change did not alter the effect of translocation intermediate on the electrical potential (as assayed by flow dialysis and oxonol VI fluorescence; data not shown). The ΔH induced by the electron transport chain was about 60 mV and not affected by the translocation intermediate. These data argue that our observed loss of Δψ upon translocation is not due to proton movement, but rather to the movement of other ion(s).

Dissipation of the Electrical Potential Is Dependent on Halide Anions—Systemic variation of the ionic composition of translocation reactions showed that the dissipation of the electrical potential by translocation intermediate depends on halide anions. Membrane vesicles were incubated with or without proOmpA-BPTI and concentrated by centrifugation. The vesicles were resuspended in a small volume of Tris-sulfate buffer and diluted with buffers containing various concentrations of chloride salts. The vesicles were equilibrated with the new buffer by bath sonication and incubation at 0 °C. The electrical potential was measured in response to NADH (Fig. 6). Dissipation of the electrical potential by translocation intermediate required at least 5 mM chloride and increased up to 15 mM chloride. Thus, the 30–50% loss of Δψ induced by translocation intermediates (Figs. 1, 3, and 4) not only depends on the chloride salt concentration, but is in addition to an already substantial dissipation of Δψ caused by the chloride salt alone. This dissipation of the potential was not caused by the different ionic strengths of the buffer solutions, since the potential was not influenced by the addition of 33 mM potassium sulfate in the absence of chloride (data not shown).

We tested whether other ions affect the electrical potential in response to a translocation intermediate (Table I). A clear selectivity for chloride, bromide, and iodide was observed, while fluoride showed a small effect. Sulfate and the different cations tested did not support translocation intermediate reduction of the electrical potential.

Electrochemical Potential-driven Translocation Is Not De-
Electrochemical potential-driven translocation of $I_{16}$ is not dependent on chloride ions. Membrane vesicles (100 $\mu$g/ml) were incubated in 50 mM HEPES-KOH, pH 8.0, 50 mM KCl, 5 mM MgCl$_2$, 2 mM DTT, 0.2 mg/ml BSA, 5 mM creatine phosphate, 10 $\mu$g/ml creatine kinase, 15 $\mu$g/ml SecB, 40 $\mu$g/ml SecA, and [35S]proOmpA for 2.5 min at 37°C. The translocation reaction was started by the addition of 2 $\mu$M ATP. After 2.5 min at 37°C, the reaction was stopped by chilling on ice. Membranes bearing the translocation intermediate $I_{16}$ (Schiebel et al., 1991) were centrifuged through a sucrose solution (0.2 M sucrose, 50 mM Tris-SO$_4$, pH 7.5, 10 mM MgSO$_4$, 1 mM DTT). The vesicles were resuspended in an equal volume of buffer C (50 mM Tris-SO$_4$, pH 7.5, 10 mM MgSO$_4$, 2 mM DTT) containing 25 mM K$_2$SO$_4$ (lanes 1–6) or 25 mM KCl (lanes 7–12). Vesicles were bath sonicated for 10 s, equilibrated with the buffer for 1 h at 4°C, and then incubated for 5 min at 37°C. Sucinate (5 mM) was added, and samples were withdrawn at 0, 20, 40, 60, 90, and 250 s. The reactions were stopped by chilling on ice. Samples were incubated with proteinase K, concentrated by trichloroacetic acid precipitation and analyzed by SDS-PAGE and fluorography.

**Translocation Induces Halide Anion Permeability**

The translocation of preproteins such as proOmpA across the E. coli plasma membrane requires catalysis by only one enzyme, the multisubunit "preprotein translocase." Translocase consists of the peripheral membrane protein SecA and the integral membrane protein SecY/E (Brandage et al., 1990). The $\Delta_{\text{H}^+}$-driven part of the translocation reaction does not require ATP or SecA (Schiebel et al., 1991).

It is not known how the electrochemical potential drives translocation. Either component of the electrochemical potential, $\Delta_{\text{H}^+}$ or $\Delta_{\nu}$, can chase $I_{16}$, a translocation intermediate in which approximately half of the proOmpA polypeptide chain has crossed the membrane, to the fully translocated species. This potential-driven chase requires neither ATP nor even the presence of SecA protein (Schiebel et al., 1991). In an attempt to understand the electrochemical potential-driven translocation, we explored whether the translocation of preprotein consumes either component of the electrochemical potential.

Our results show that a translocation intermediate can, in the presence of halide anions, contribute to the dissipation of the electrochemical potential imposed by the electron transport chain. Certain possible indirect effects could be excluded, such as an inhibition of the NADH oxidase by the translocation intermediate or an uncoupling effect due to a leader peptide. A clear correlation between the presence of a translocation intermediate and this dissipation of the electrical potential was found. However, $\Delta_{\text{H}^+}$ was not affected by the translocation intermediate, making it unlikely that we were observing proton movement in response to the translocation intermediate. Additional experiments established that preprotein translocation enhances the already substantial halide anion permeability of the E. coli plasma membrane, which explains the dissipation of $\Delta_{\nu}$ but not $\Delta_{\text{H}^+}$. To determine whether the particular membrane-spanning sequence of proOmpA influences the halide anion conductivity, further studies, with proOmpA translocation intermediates arrested at different stages of the translocation reaction, will be required.

It is clear that halide anions are not necessary for translocation. The observed anion conductance, while a result of protein translocation, is not required for the translocation of proteins. Nevertheless, this halide anion permeability can be seen in the context of the old question of whether preproteins cross a membrane through the lipid phase or through a protein "pore." proOmpA may cross the plasma membrane of E. coli via the lipid, along the interface between lipid and SecY/E, or through a central domain of the multi-spanning SecY/E protein where it would be shielded from the fatty acyl chains. Our studies of the interaction of the translocation intermediate with the membrane potential have not, by themselves, answered this question, but provide an interesting window to view the mechanism of translocation. If proteins were to translocate through a protein pore, our results might suggest that halide anions could pass between the translocating protein and the "walls" of this pore. This flexibility of the pore would be necessary to allow the various amino acyl side chains to pass through a common site. In this model, the basis of the ion selectivity is not clear, though transport channels which can conduct the various halide anions have been reported (Coronado and Latorre, 1982; White and Miller, 1979). Translocation pores have been implicated in previous studies. Simon et al. (1989) fused E. coli membrane vesicles to black lipid membranes and detected channels of 115 picosiemens. However, since translocating preproteins were not provided, these 115-picoseiemen channels are unlikely to be related to the halide anion conductance reported here.

We find that the translocation intermediate-induced halide anion permeability follows the same order of ion specificity as the ability of these ions to collapse a $\Delta_{\nu}$ across membrane vesicles (without translocation intermediates) or across proteoliposomal bilayers. This suggests that the induced halide anion permeability is across the lipid phase of the membrane per se rather than through a protein such as SecY/E. This is most simply explained by the preprotein crossing the membrane in contact with the lipid phase and changing the lipid structure in such a way that the permeability of halide ions is increased. In both models the driving force for the influx of the halide ions is the imposed electrochemical potential, inside positive.

**Acknowledgments**—We thank Douglas Geissert and Marilyn Rice for expert technical assistance. We thank Dr. Martine Bassilana and Dr. Ronald Kaback for stimulating discussions.

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