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Role for membrane potential in the secretion of protein into the periplasm of *Escherichia coli*

(leucine-specific binding protein/*in vivo* accumulation of precursor proteins)

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ABSTRACT **The leucine-specific binding protein of *Escherichia coli* is a periplasmic protein that is synthesized as a precursor and subsequently is processed during its secretion into the periplasmic space. The processing of both the leucine-specific binding protein and a plasmid-coded β-lactamase is inhibited by phenethyl alcohol and by the proton ionophore, carbonylcyanide *m*-chlorophenylhydrazone (CCCP). The levels of CCCP that inhibit processing also produce significant decreases in the membrane potential. Valinomycin, a potassium ionophore, also inhibits processing of the leucine-specific binding protein in spheroplasts. Processing can be restored in CCCP-treated cells and in valinomycin-treated spheroplasts by dilution of the treated cells in fresh medium. These results suggest a role for membrane potential in the secretion of periplasmic proteins. A model is presented which suggests that membrane potential plays a primary role in the proper orientation of the precursor signal sequence within the membrane, thus promoting processing and secretion.**

Escherichia coli periplasmic proteins are synthesized on membrane-bound ribosomes in precursor forms with an NH_2-terminal amino acid sequence (signal sequence) and subsequently are processed and secreted through the inner membrane (1, 2). During the secretory process, the signal sequence (3) is cleaved by a leader peptidase (4). We have shown that the leucine-specific binding protein, which is a periplasmic component of a leucine transport system in *E. coli*, is synthesized as a precursor that contains a 23-amino acid signal sequence (5). Using pulse–chase experiments with intact cells, we recently have found that the precursor of the leucine-specific binding protein can be processed after translation (unpublished data). Although the structure of a number of signal sequences have been determined (6), the mechanism of membrane translocation of the nascent protein is not understood. A role for membrane potential has been implicated for the proper insertion and processing of the M13 precoat protein within the cytoplasmic membrane (7, 8). In this report, we demonstrate a requirement for a membrane potential in the processing and secretion of two *E. coli* periplasmic proteins, the leucine-specific binding protein and β-lactamase. During the preparation of this manuscript, we learned of studies in Linda Randall's laboratory showing that the export of several *E. coli* outer membrane proteins (products of *ompF*, *ompA*, and *lamB* genes) and the periplasmic binding proteins for maltose and arabinose also require a membrane potential (9, 10).

METHODS

CCCP Treatment. The *E. coli* strains, AE191, (*lstR*, *livJ*, *livP9*, *arg*, *his*, *trp*, *thy*, *leu*) and the Hfr Hayes Δ*lon* strain (P. Bassford collection) were used in these experiments. These strains were transformed (11) with the hybrid plasmid pOX7 (5), which carries the *livK* gene coding for the leucine-specific binding protein and the *bla* gene for β-lactamase. These transformed strains were grown in 4-morpholinepropanesulfonic acid (Mops)-rich medium (12) in the absence of methionine and leucine. The pOX7 plasmid was maintained in these strains by including ampicillin (20 μg/ml) in the growth medium. The effects of phenethyl alcohol (2-phenylethanol; PhEtOH) and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were examined by first growing cells to an A_{600} of 0.8. The cells were harvested by centrifugation and resuspended to their original density in 100 mM Tris·HCl, pH 6.8/10 mM EDTA. This cell suspension was incubated at 37°C for 5 min, centrifuged, and then resuspended to their original density in Mops-rich medium at 23°C. PhEtOH or CCCP was added to 100 μl of cell suspension. After 10 min of incubation, the suspension was shifted to 37°C, and 12 μCi of L-[^{35}S]methionine was added. Incubation was continued for 30 min at 37°C. Labeled proteins from whole cells and immunoprecipitates were separated on $NaDodSO_4$/12% polyacrylamide gels as described by Laemmli (13).

Valinomycin Treatment. The effects of valinomycin were examined in spheroplasts of strain AE191/pOX7. Spheroplasts were prepared as described by Osborn *et al.* (14). The spheroplasts were resuspended to 20% of the original culture volume in Mops-rich medium (minus methionine) containing 12% (wt/vol) sucrose. Valinomycin was added to 100 μl of the spheroplast suspension at 23°C, and the incubation was continued for 10 min. The spheroplast suspension was shifted to 37°C, and 12 μCi of L-[^{35}S]methionine was added. Incubation was continued for 30 min at 37°C, followed by immunoprecipitation and analysis as described (13).

Reversal of Inhibition of Processing. The restoration of processing was examined in cells treated with PhEtOH and CCCP and in valinomycin-treated spheroplasts. After treatment of the cells with either PhEtOH or CCCP, the cells were centrifuged and resuspended in Mops-rich medium without the inhibitor. Incubation (60 min) of the suspension was followed by centrifugation, immunoprecipitation, and analysis by electrophoresis. Spheroplasts of strain AE191/pOX7 were treated with 40 μM valinomycin and labeled as before. Spheroplasts were centrifuged and resuspended to their original density in 125 mM Tris·HCl (pH 6.8) containing 12% sucrose, 5 μM valinomycin, and various concentrations of KCl. Incubation of the suspensions at 37°C for 60 min was followed by immunoprecipitation and analysis by electrophoresis.

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Mops, 4-morpholinepropanesulfonic acid; PhEtOH, phenethyl alcohol (2-phenylethanol); Ph_3MePBr, triphenylmethylphosphonium bromide; $\Delta\Psi$, calculated membrane potential.

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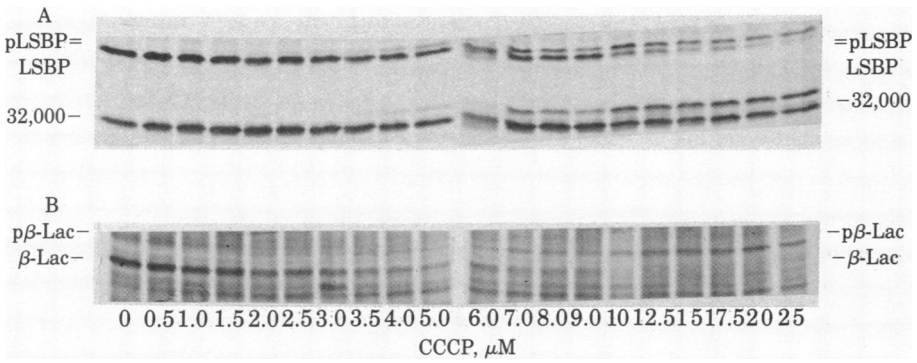

FIG. 1. Effects of CCCP on the processing of the leucine-specific binding protein and β-lactamase precursors. Half of the CCCP-treated and labeled cells were subjected to immunoprecipitation followed by $NaDodSO_4$/polyacrylamide gel electrophoresis to identify the leucine-specific binding protein and its precursor (*A*). The remaining cells were prepared for direct analysis by electrophoresis (*B*). The gels were stained, destained, dried, and autoradiographed. The positions of the leucine-specific binding protein (LSBP), its precursor (pLSBP), the 32,000 molecular weight protein, β-lactamase (β-Lac), and its precursor (pβ-Lac) are indicated.

Measure of Membrane Potential. Changes in the membrane potential during CCCP treatment were examined by measuring the uptake of ^3H-labeled triphenylmethylphosphonium bromide (Ph_3MePBr) (15). Cells were grown as described and resuspended in 30 mM sodium phosphate, pH 6.8/10 mM EDTA. After a 5-min incubation at 37°C, the cells were isolated and resuspended in 30 mM sodium phosphate, pH 6.8/0.2% glucose at 23°C. Various concentrations of CCCP in 1% (final concentration) ethanol were added to the cells. After 5 min of incubation at 23°C, 100-μl aliquots of the cell suspensions were shifted to 37°C, and 9.1 nCi (1 Ci = 3.7×10^{10} becquerels) of $[^3H]Ph_3MePBr$ (3.59 Ci/mmol) was added to each sample. Incubation of the suspensions at 37°C for 10 min was followed by centrifugation for 3 min in an Eppendorf model 3200 centrifuge at 23°C. The radioactivity in the supernatant fluids and cell pellets was measured by liquid scintillation spectrometry. In parallel experiments, $[^3H]H_2O$ (1 mCi/g) and [^{14}C]dextran (2.76 mCi/g) were added to measure total and extracellular water space, respectively. Uptake of Ph_3MeP^+ was expressed as pmol/mg of protein. Protein was measured by the method of Lowry *et al.* (16). The apparent membrane potential ($\Delta\Psi$) was calculated from the Nernst equation ($\Delta\Psi = 58.8 \log[Ph_3MeP^+]_{in}/[Ph_3MeP^+]_{out}$) using the steady-state levels of $[^3H]Ph_3MeP^+$ (17).

RESULTS

Effects of CCCP on Processing of Periplasmic Proteins. When the *E. coli* strains AE191 and Δ*lon* were transformed with the plasmid pOX7, the leucine-specific binding protein and β-lactamase became major periplasmic protein products. A detailed examination of the action of CCCP on the processing of these proteins is presented in Fig. 1. For this study, cells of the Δ*lon*/pOX7 strain were incubated in Mops-rich medium with various concentrations of CCCP at 23°C for 5 min and then shifted to 37°C for a 30-min labeling period with 12 μCi of L-[^{35}S]methionine. The amount of mature forms of the leucine-specific binding protein and a M_r 32,000 Triton X-100-insoluble protein, designated 32,000 M_r protein, decreased with increasing levels of CCCP (Fig. 1*A*). Concomitantly, precursor forms of the leucine-specific binding protein and the M_r 32,000 and protein appeared as the level of CCCP was increased. Physicochemical properties of the M_r 32,000 protein were similar to those reported for the outer membrane protein product of *ompA* gene (9). β-Lactamase exhibited a similar response to CCCP treatment (Fig. 1*B*). Similar results were obtained with strain AE191/pOX7.

Measurement of Membrane Potential During CCCP Treatment. Although the processing of all three of these proteins was inhibited by CCCP, the proteins differed markedly in their sensitivities (Fig. 2*A*). Processing of β-lactamase was most sensitive, then processing of the leucine-specific binding protein, and finally processing of the M_r 32,000 protein.

The observed inhibition of processing of periplasmic proteins occurred at CCCP concentrations that have been reported to dissipate the membrane potential of *E. coli* (17). To examine changes in the apparent membrane potential, we measured the uptake of Ph_3MeP^+ (17) during the treatment of cells with CCCP. Addition of 1–5 μM CCCP caused a significant decrease

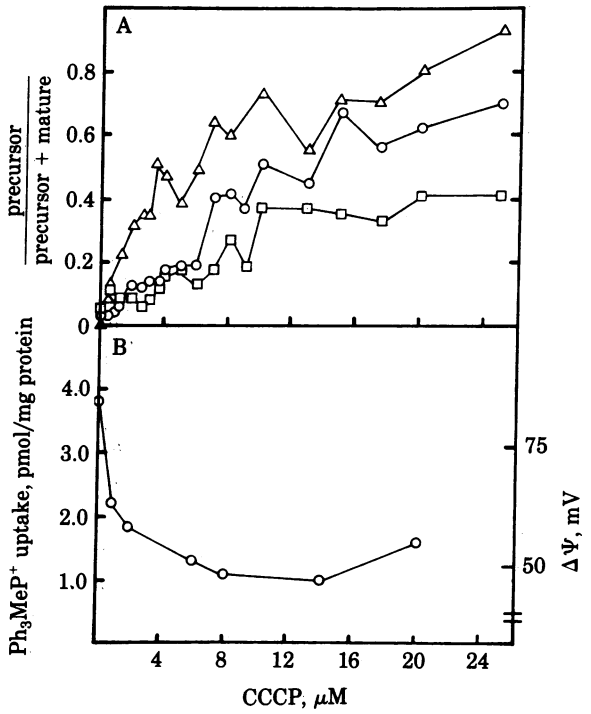

FIG. 2. The effect of CCCP on the processing of precursor proteins and the uptake of $[^3H]Ph_3MeP^+$. Cells of strain Δ*lon*/pOX7 were labeled in the presence of CCCP. The labeled proteins were analyzed by electrophoresis. The band for the precursor and processed forms of β-lactamase, leucine-specific binding protein, and M_r 32,000 were cut from the dried gels and rehydrated for 4 hr at 37°C in 8 ml of liquid scintillation mixture, and the radioactivity was measured. (*A*) Fraction of the total radioactivity represented by the precursor for each protein—leucine-specific binding protein (○), M_r 32,000 protein (□), and β-lactamase (△)—at increasing CCCP concentrations. (*B*) Effect of CCCP on uptake of $[^3H]Ph_3MeP^+$ and membrane potential. Cell pellets and supernatants isolated as described were analyzed for $[^3H]Ph_3MeP^+$. Total water in the cell pellet was measured with $[^3H]H_2O$ and extracellular water was measured with [^{14}C]dextran. Membrane potential was calculated from the equilibrium distribution ratio $[Ph_3MeP^+]_{in}/[Ph_3MeP^+]_{out}$ using the Nernst equation.

in Ph_3MeP^+ uptake (Fig. 2*B*). The steady-state distribution of Ph_3MeP^+ was used to calculate an apparent membrane potential ($\Delta\Psi$) from the Nernst equation. The calculated membrane potential decreased from −85 to −45 mV over the range of CCCP concentrations shown (Fig. 2*B*).

Reversal of Inhibition of Processing. We attempted to reverse the inhibition of processing caused by PhEtOH and CCCP by resuspending the treated cells in media free of inhibitor. Inhibition of processing produced by either compound could be completely reversed during a 60-min incubation in the absence of inhibitor (Fig. 3*A*). The addition of chloramphenicol (Fig. 3*A*, lanes d and h) did not alter the amount of mature leucine-specific binding protein during this incubation, showing that continued protein synthesis was not responsible for the appearance of the mature leucine-specific binding protein. When PhEtOH-treated cells were resuspended in a medium containing 32 μM CCCP, restoration of processing was partially inhibited (Fig. 3*A*, lane e). The addition of PhEtOH also prevented the restoration of processing in CCCP-treated cells (Fig. 3*A*, lane j). Fig. 3*B* shows the cellular location of the mature leucine-specific binding protein after the restoration of processing in PhEtOH- and CCCP-treated cells. After each incubation of the treated cells in an inhibitor-free medium (Fig. 3*A*, lanes c, d, g, and h), an equal aliquot was subjected to osmotic shock treatment. An analysis of the immunoprecipitate of the shock fluids and the cell pellets showed that restoration of processing leads to a quantitative conversion of the precursor to the mature form of the leucine-specific binding protein and to its secretion into the periplasm (Fig. 3*B*, lanes l, n, p, and r).

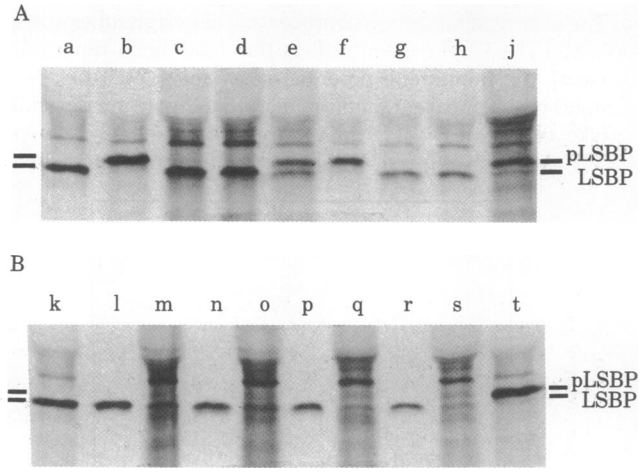

FIG. 3. Reversibility of the inhibition of processing and secretion of the leucine-specific binding protein (LSBP) after treatment of cells with PhEtOH and CCCP. (*A*) Inhibition of processing and its reversal. (*B*) Cellular location of the LSBP resulting from the reversal. Cells of strain AE191/pOX7 were suspended in Mops-rich medium without methionine and incubated (with no additions) with 0.42% PhEtOH or with 32 μM CCCP at 23°C for 10 min. The cells were then pulse-labeled for 30 min at 37°C with L-[^{35}S]methionine. At the end of the labeling period, the untreated cells (lanes a and k), samples of the PhEtOH-treated cells (lanes b and t), and samples of CCCP-treated cells (lane f) were removed for immunoprecipitation and electrophoresis. To measure the reversal of inhibition of processing, the remaining suspensions were centrifuged, and the cells were resuspended in Mops-rich medium with various additions and incubated for 60 min at 30°C. At the end of the incubation, the samples were halved for analysis. One half of each sample was immunoprecipitated and analyzed by electrophoresis (*A*). Cells from the remaining half were subjected to osmotic shock treatment (18), and the shock fluids and cell pellets were analyzed by immunoprecipitation and electrophoresis (*B*). The position of the leucine-specific binding protein (LSBP) is indicated by the lower bars and by the band in lanes a and k. The position of the precursor to the LSBP (pLSBP) is indicated by the upper bars and by the position of the protein band in lanes j and t. Other lanes: b, inhibition of processing by 0.42% PhEtOH; c, reversal of PhEtOH inhibition of processing; l and m, immunoprecipitates of the shock fluid and cell pellet, respectively, corresponding to lane c; d, reversal of PhEtOH inhibition in the presence of chloramphenicol (100 μg/ml); n and o, the shock fluid and cell pellet corresponding to lane d; e, addition of 32 μM CCCP during reversal of PhEtOH inhibition; f, inhibition of processing by 32 μM CCCP; g, reversal of CCCP inhibition; p and q, shock fluid and cell pellet corresponding to lane g; h, reversal of CCCP inhibition in the presence of chloramphenicol (100 μg/ml); r and s, shock fluid and cell pellet corresponding to lane h; j, addition of 0.42% PhEtOH during reversal of CCCP inhibition.

The Effect of Valinomycin on Processing. We investigated the action of valinomycin, a potassium ionophore that disrupts membrane potential (19), on the processing of bacterial periplasmic proteins. Because valinomycin does not readily penetrate the outer membrane of *E. coli*, we used spheroplasts in these experiments. The processing of the leucine-specific binding protein in spheroplasts was completely blocked by the presence of 5 μM valinomycin (Fig. 4*A*). The membrane potential in valinomycin-treated spheroplasts could be partially restored by diluting the external potassium, thus recreating a potassium gradient. Resuspension of the valinomycin-inhibited spheroplasts in K^+-free buffer partially reversed the inhibition and permitted significant processing to occur. The addition of increasing levels of potassium ion to the buffer prevented the reversal of inhibition of processing.

DISCUSSION

The results of our studies suggest that membrane potential plays a role in the secretion of periplasmic proteins in *E. coli*. The addition of a proton ionophore, CCCP, at concentrations that dissipate the membrane potential can prevent the processing of several periplasmic proteins, including the leucine-specific binding protein and β-lactamase. Valinomycin, a potassium ionophore, also inhibits processing of the leucine-specific bind-

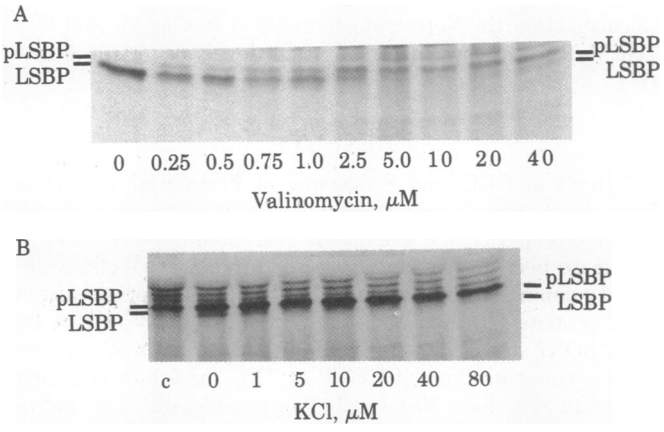

FIG. 4. Effects of valinomycin on the processing of the leucine-specific binding protein precursor (pLSBP) (*A*). Cells of strain 191/pOX7 were grown in Mops-rich medium and spheroplasts were prepared, treated with valinomycin and labeled with L-[^{35}S]methionine as described. Immunoprecipitates were analyzed by electrophoresis; positions of LSBP and pLSBP are indicated. (*B*) Restoration of processing activity in the presence of valinomycin. At the end of the labeling incubation, a sample of the spheroplasts was removed for immunoprecipitation (lane c). The remaining spheroplasts were isolated by centrifugation and resuspended in 125 mM Tris·HCl (pH 6.8) containing 5 μM valinomycin and various concentrations of KCl. The suspension was shifted to 37°C, and the incubation was continued for 60 min. After the incubation, the labeled spheroplasts were analyzed for LSBP and pLSBP by immunoprecipitation, followed by gel electrophoresis; positions of LSBP and pLSBP are indicated.

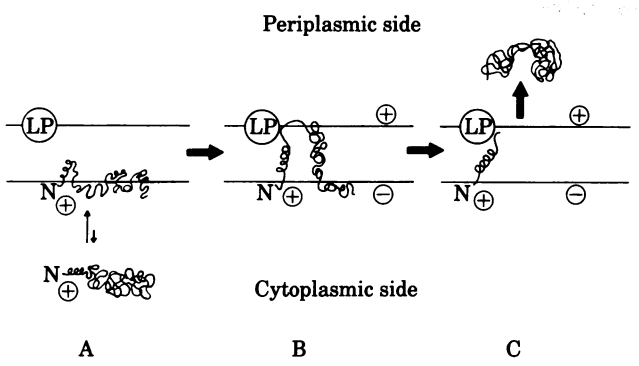

FIG. 5. Model for secretion of periplasmic proteins.

ing protein. Partial restoration of processing and secretion of the leucine-specific binding protein was observed when the treated cells were resuspended in inhibitor-free medium. It is unlikely that the CCCP treatment inhibits the leader peptidase itself because Wickner and colleagues (7) have shown that purified processing enzyme is not inhibited by CCCP, and we have found that samples of this enzyme (provided by Wickner) process the leucine-specific binding protein precursor synthesized *in vitro* (20). Our observation that β-lactamase and the leucine-specific binding protein respond to inhibition in a quantitatively different manner also suggests that cleavage of the precursors by the leader peptidase is not the membrane potential-dependent step because this step is assumed to be common in the processing of both proteins.

In previous reports, Wickner and his colleagues have shown that CCCP prevents processing of the M13 coat protein and concluded that a membrane potential was necessary for proper insertion of the coat protein into the membrane and the subsequent processing of its precursor form (7, 8). Recent studies in L. Randall's laboratory done in parallel with our studies also have shown that membrane potential is essential for the secretion of several *E. coli* periplasmic and outer membrane proteins (9). The processing and secretion of periplasmic proteins in *E. coli* involves several steps, and it is not clear which step or steps require(s) a membrane potential.

For purposes of discussion we will refer to the model shown in Fig. 5. This model has several features similar to the one proposed by Wickner and coworkers (8). However, we have attempted to extend these features to include a role for membrane potential in the transport of periplasmic proteins. In our proposed model, the principal role of the membrane potential is to orient the precursor within the membrane so that it will be accessible to the leader peptidase. Fig. 5*A* suggests that, in the absence of a membrane potential, the precursor will distribute itself between the cytoplasm and the membrane. This distribution will depend on the relative hydrophobicity of the precursor. We have found that the precursors of the leucine-specific binding protein and β-lactamase were largely associated with the membrane. When a membrane potential is present, as shown in Fig. 5*B*, the signal sequence would be correctly oriented within the membrane, where it would be accessible to the leader peptidase. This orientation could be accomplished by the alignment of the dipoles and charged groups of the precursor with the electric field across the membrane. The presence of positive charges near the NH_2-terminus in some cases may serve as an anchor on the cytoplasmic side of the membrane as suggested by Inouye and colleagues (21). After cleavage of the signal sequence, the mature form of the peptide would spontaneously refold in a conformation that is incompatible with its solubility in the membrane. The energy of refolding and the membrane potential may be responsible for the vectorial translocation of the protein into the periplasmic space.

According to this model, more of the polypeptide chain than the signal sequence may be required to provide the folding energy necessary for secretion of the processed form into the periplasm. The improper refolding of a chain-terminated protein may cause it to either stay in the membrane or pull back out into the cytoplasm after cleavage of the signal sequence. This may explain why some incomplete β-lactamase polypeptides are processed but found in the cytoplasm as reported by Koshland and Botstein (22). This model can also account for the variable sensitivities of precursor processing to uncouplers because the electric field required to properly orient each precursor may differ. The primary feature of this model is the role of the membrane potential in the proper orientation of a portion of the polypeptide chain within the membrane.

Thermodynamic considerations of how electrical fields alter the orientation of dipoles and charged groups of small molecules (23) and of polypeptides (24) have been discussed. A suggested functional role of the membrane potential in the induction of new conformational states of polypeptide chains within the membrane is provided by studies of an eicosapeptide, alamethicin, which forms voltage-dependent channels in phospholipid bilayer membranes (25). In addition, Blumenthal *et al.* (26) have demonstrated a voltage-dependent translocation of asialoglycoprotein receptors in lipid bilayers, and Donovan *et al.* (27) have shown that diphtheria toxin forms voltage-dependent, anion-selective channels. Collectively, these studies indicate a general role for membrane potential in the functional orientation of polypeptides within the membrane.

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