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Signal Peptides Open Protein-Conducting Channels in E. coli

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Summary

Plasma membrane vesicles and protoplasts of Escherichia coli were fused to planar lipid bilayers and studied with electrophysiological techniques. Large transmembrane aqueous channels were opened when 0.2 nM LamB signal peptide was added to the cytoplasmic side of the membrane. These aqueous pores are similar in conductance to those previously observed in mammalian endoplasmic reticulum when puromycin is used to release and thus unplug nascent translocating chains. Signal sequences have been previously shown to be necessary and sufficient for targeting proteins to cellular membranes. These results demonstrate that signal peptides are sufficient for opening the protein-conducting channels. We suggest that they are the physiological ligands that open protein-conducting channels at the initiation of protein translocation across prokaryotic plasma membrane and mammalian endoplasmic reticulum.

Introduction

How proteins cross or are integrated into membranes is an important issue in cellular growth, secretion, and organelle biogenesis. Whether proteins translocate through the hydrocarbon core of the bilayer or through transmembrane aqueous channels has been a key mechanistic question. We have recently obtained evidence supporting the existence of protein-conducting channels. Specifically, large aqueous channels were observed in the endoplasmic reticulum (ER) when nascent translocating peptides were released from their membrane-bound ribosomes (Simon and Blobel, 1991). In low salt concentrations (50 mM KCl), these channels have a conductance of 220 pS. When the salt concentration is subsequently raised to higher than physiological concentrations (>150 mM, which dissociates the ribosomes from the membrane) these channels close.

These results are consistent with the idea that translocation across the mammalian ER membrane occurs through protein-conducting channels. While translocating a protein, these channels are open but electrically silent, i.e., they are unable to conduct ions freely when plugged with a translocating chain. Upon release of the proteins, the channels are unplugged and thus freely conductive to ions. Association of the ribosome with the translocation apparatus helps stabilize these channels in an open conformation.

At least three questions are immediately suggested. First, can alternative evidence be found to test for the presence of protein-conducting channels that does not depend on the use of puromycin? Second, as closure of the channel seems to be tightly coupled to the termination of protein translation–translocation, how is the opening of the channel coupled to the initiation of translocation? Third, how general are protein-conducting channels as a mechanism for translocating proteins?

We have addressed all three issues by asking whether signal peptides were sufficient for opening such channels in the Escherichia coli plasma membrane. There were a number of advantages to this experimental approach. The signal sequences that target proteins to the mammalian ER and the E. coli plasma membrane are highly interchangeable. Thus, translocation across the E. coli plasma membrane may share other elements, such as protein-conducting channels. Additionally, translocation of proteins across the E. coli membrane is not obligatorily co-translational. Thus, in E. coli, it should be possible to examine the potential roles of the signal sequence and of cytosolic factors independently of the presence of ribosomes. Channel opening was expected to be coupled with the initiation of translocation. As the channels open, they would be occupied by the nascent translocating chain and hence blocked. If the signal for opening the channels did not reside in the mature translocating chain, it might be possible to dissociate channel opening from chain insertion and measure the flow of ions through the channel.

In this paper, we demonstrate that adding signal peptides to the cytoplasmic face of the E. coli membrane opens channels of ~220 pS (in 50 mM KCl) in the E. coli membrane. These channels are indistinguishable in conductance from the puromycin-revealed channels in the ER (Simon and Blobel, 1991). Three conclusions are drawn. First, the observation that signal peptides, physiologically relevant ligands, can gate open large aqueous pores corroborates the puromycin results. Taken together, these results provide compelling evidence for protein-conducting channels in cellular membranes. Second, the signal sequence is the physiological ligand that opens the protein-conducting channel. Third, protein-conducting channels exist in both prokaryotes and eukaryotes.

Results

The conductance properties of E. coli plasma membranes were studied by fusing them to a planar lipid bilayer that separates two compartments. All membranes were added to the cis chamber, which was kept hyperosmotic to the trans chamber. The topography of membranes is maintained when they are fused with the bilayer. Thus, the surface of the vesicle that faces inward will, after fusion, face the solution bathing the trans chamber. The conductance was assayed by applying a constant voltage across

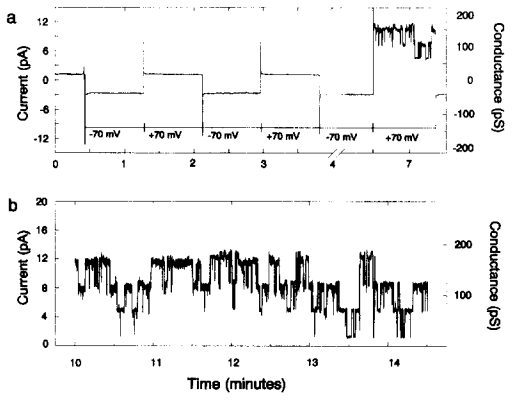

Figure 1. Fusion of InV to Planar Lipid Bilayer

(a) A planar lipid bilayer was voltage clamped to pulses of +70 and −70 mV in the presence of 50 mM KCl and 5 mM K–HEPES (pH 7.5). The transient current flow during the change of potential is the current used to charge the capacitance of the system. InV were fused to the bilayer during the break in the record between 4 and 6 min (the solutions were being vigorously stirred, which produced, at this amplification, a very noisy current recording). Upon fusion of InV to the membrane, there is only a subtle increase of bilayer conductance at −70 mV. In contrast, there is a pronounced increase at +70 mV (6.5–7.4 min) where a 60 pS channel can be clearly observed.

(b) When the voltage across the membrane was maintained at +70 mV, the 60 pS channels continuously opened and closed. Three distinct conductance levels can be distinguished. The fusion was induced by an osmotic gradient of 300 mM glycerol (cis to trans). At the first sign of fusion, the osmotic gradient was equilibrated by the addition of glycerol to the trans chamber.

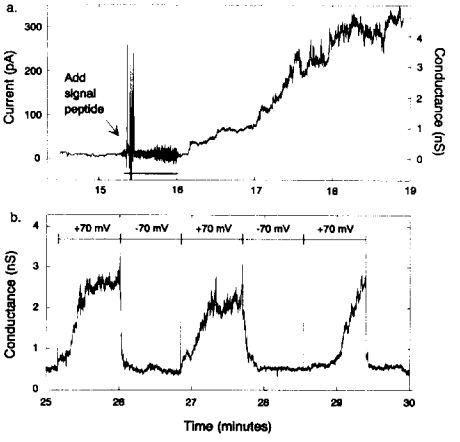

Figure 2. Signal Peptides Elicit a Large Increase of Membrane Conductance

This is a continuation of the same experiment shown in Figure 1.

(a) A 2 μl aliquot of 400 μM LamB signal peptide (in 8 M urea) was added to the cis compartment bathing the bilayer where indicated (200 nM final concentration). The horizontal bar indicates the period during which the solutions were continuously stirred. After the addition of LamB signal peptide, the membrane conductance stabilized at 4.5 nS. The membrane potential was maintained at +70 mV throughout this tracing.

(b) The membrane potential was changed, where indicated, back and forth between +70 and −70 mV. This record is a continuation of the experiments shown in Figures 1a and 1b and Figure 2a. As the voltage was changed from one polarity to another, there was a transient capacitive charging of the membrane.

the membrane and measuring the electrical current. This approach allows easy electrophysiological control over the membranes and allows the solutions bathing both surfaces of the membrane to be independently changed. We have fused three different kinds of E. coli membrane preparations: protoplasts (with the cytoplasmic surface facing inward); inverted vesicles of plasma membrane (cytoplasmic surface facing outward); and outside-out vesicles of the plasma membrane. The results obtained from the three preparations were indistinguishable (data not shown).

The current flowing across a plain lipid bilayer in response to voltage pulses of 50 mV (alternating positive and negative) is shown in Figure 1a (0–4 min) for a lipid bilayer of 1 mm^2 in a solution of 50 mM KCl and 5 mM K–HEPES (pH 7.5). Inverted vesicles of the E. coli plasma membrane (InV) were injected onto the cis surface of the bilayer at 4 min while both chambers were vigorously stirred. At the first sign of a change of membrane conductance—indicating membrane fusion—the osmotic gradient was eliminated to prevent additional fusion. When the cytosolic surface was negative (−70 mV)—the normal in situ condition (from 6 to 6.5 min)—the conductance was very similar to that of the plain bilayer at −70 mV (compare with 0 to 4 min). (All voltages will be described for the cytosolic side relative to the periplasmic side, which was held at ground). Very few rather small channels could be observed. These are potassium channels but they have not been carefully examined and will not be further characterized in this paper. Thus, at physiological membrane potentials, the E. coli plasma membrane, like eukaryotic plasma membranes, is rather impermeant to the free flow of ions. However, at +70 mV, a distinct 55–60 pS channel was consistently observed (Figure 1a, 6.5–7.3 min). When the same membrane was maintained at a +70 mV, these channels continuously opened and closed (Figure 1b). In addition, as previously observed, a 115 pS channel was occasionally observed that also opened at positive membrane potentials (data not shown and Simon et al., 1989).

Signal Peptides Increase Membrane Conductance

Adding 200 nM signal peptide to the cytoplasmic surface of this same membrane yielded a substantial increase in membrane conductance at +70 mV (Figure 2a). To facilitate a comparison of the magnitude of this conductance, the channels shown in Figure 1b (10–14.5 min) are the same as those seen in the first 1 min of Figure 2 (14–15 min). At +70 mV, this conductance reached a steady state. When the membrane potential is −70 mV, the conductances quickly decreased (Figure 2b from 26–26.85 min or 27.7–28.55 min). Upon return to a positive membrane potential, the conductances increased again (26.85–27.7 min or 28.55–29.4 min). This process is completely and continuously reversible.

It has been demonstrated that, at 100 μM concentrations, some signal peptides can interact directly with lipid bilayers (Killian et al., 1990). However, when 10 μM signal peptide was added directly to plain lipid bilayers in the absence of E. coli membranes, there was no effect on membrane conductance (Figure 3).

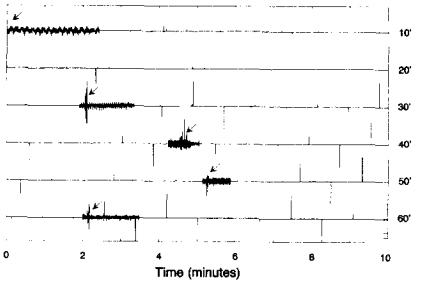

Figure 3. LamB Signal Peptide Does Not Affect the Conductance of Lipid Bilayers

A lipid bilayer was formed in the hole of a Teflon partition separating two compartments of 4 ml of 50 mM KCl and 5 mM K–HEPES (pH 7.5). These six traces are a continuous record of 1 hr duration (the elapsed time is indicated on the right). At each arrow, 10 μl of 1 mM LamB signal peptide was added to the solutions where indicated. Even after the addition of 50 μl (12.5 μM final concentration) and stirring for 1 hr, there was no observable effect on membrane conductance. The experiment was continued for an additional 50 min with four more additions of 10 μl of 1 mM signal peptide with the same results. The small deflections up and down from the baseline are capacitive transients from changing the polarity of the membrane potential (± 70 mV). The small calibration bars in the lower righthand corner are 1 pA markings (~14 pS).

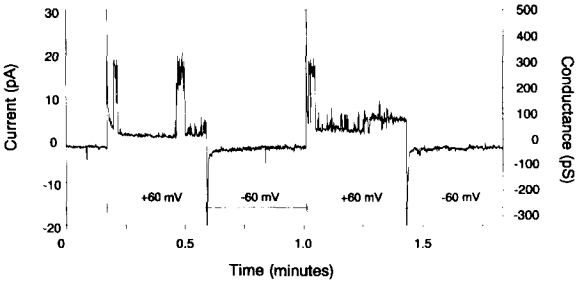

Figure 4. Individual, Large (220 pS) Channels Are Gated Open in Low (0.2 nM) Concentrations of Signal Peptide

InV were fused to a planar lipid bilayer in the presence of 50 mM KCl and 5 mM K–HEPES with an osmotic gradient of 300 mM glycerol (as described in Figure 1). The membrane potential was successively stepped back and forth −60 mV to +60 mV. At each transition to +60 mV, there is a large, brief upward transient and a downward transient at each transition to −60 mV. Two different channels can be observed of 60 and 220 pS, both of which are usually only observed at positive membrane potentials.

Signal Peptides Gate Open Single Channels

A smaller quantity of signal peptide, 0.2 nM, was used to examine the microscopic basis of the large macroscopic signal peptide–induced conductance of Figure 2a. This concentration of signal peptide is the equivalent of 1 signal peptide per 8 E. coli. After addition of signal peptide, a new channel of approximately 220 to 240 pS appeared that was open only at positive membrane potentials (Figure 4). This channel opens twice during the first step to +60 mV in Figure 4 (at ~0.24 and 0.5 min) and once during the second step to +60 mV (1.1 min). The previously observed 60 pS channel can still be observed opening primarily at positive voltages (1.2–1.3 min), although it can be observed opening very briefly with the membrane at −60 mV (at 0.15 min). Given this time, amplitude, and frequency resolution, it is impossible to distinguish any of the small potassium channels.

All the experiments described above were done in low, 50 mM salt. As shown in Figure 5, at higher salt concentrations (400–1000 mM), the signal peptide also induced increases of conductance. The magnitude of the conductance of these channels was roughly proportional to the salt concentration. Because they were being opened by the same ligand and were of the same apparent conductance, it is reasonable to assume they are the same channel. However, there were two striking differences. First, in high salt the channels opened and stayed open; in this particular example, application of signal peptide resulted in five step increases of membrane conductance (Figure 5). This is quite different from the observation in low salt where the large, signal peptide–activated channel gates open and close. Second, at high salt concentrations the channels are not closed by negative voltages.

Proteins translocate vectorially from the cytosol to the periplasm. Thus, it is reasonable to assume that the ligand-binding site for gating a protein-conducting channel open would face the cytoplasm. In the previous experiments, signal peptide was added to what was equivalent to the cytosolic side of the membrane (the cis compartment for InV and the trans compartment for outside-out membranes and protoplasts). We next tested whether signal peptides induce a conductance on only the cytoplasmic side or on both sides of the membrane. For this experiment, we used protoplasts rather than InV or outside-out vesicles. One potential concern was that the signal peptide might be inducing additional vesicles to fuse with the bilayer, even though we had eliminated the osmotic gradient used to induce fusion. There were two advantages of using

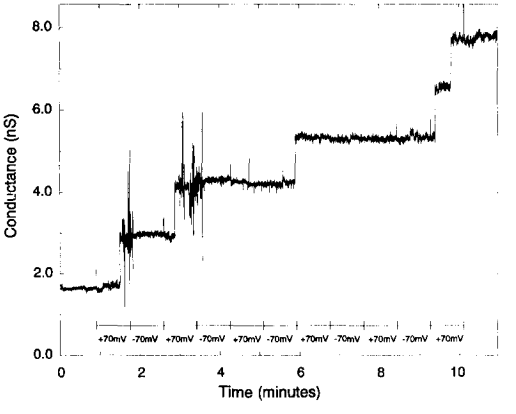

Figure 5. Signal Peptide–Gated Channel Remains Open in High Salt

Protoplasts were fused to the lipid bilayer in the presence of a KCl gradient of 700 mM. After fusion, the osmotic gradient was eliminated by raising the KCl on the trans side to 700 mM. Subsequently, 2 μl of 400 nM signal peptide was added to the trans side (at t = 1.1 min), with a final concentration of 0.2 nM. Step increases of conductance occurred at t = 1.7, 3, 6, 9.5, and 9.7 min. The line near the bottom indicates whether the membrane potential was +70 mV or −70 mV. Note that while all the channel openings occurred during positive membrane potentials, the channels remained open when the voltage was switched to −70 mV.

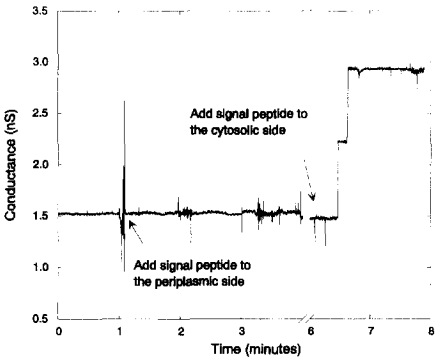

Figure 6. Signal Peptides Open Channels When Presented on the Cytosolic Side of the Membrane

Protoplasts were fused to a lipid bilayer as described in Figure 5 with an osmotic gradient of 400 mM KCl. After equilibrating the osmotic gradient, 0.2 nM signal peptide was added first to the cis (periplasmic) side of the bilayer. After 6 min, 0.2 nM signal peptide was added to the trans (cytosolic) side of the membrane.

protoplasts. First, since they were facing outside out, the signal peptide would be expected to work on the side of the membrane opposite the addition of protoplasts, where it would not be able to induce additional fusion. Second, neither of the other two membrane preparations were 100% inside out or 100% outside out and thus would not be as effective in a test of the efficacy of the signal peptide on the cis vs. trans sides. After fusion of protoplasts to a bilayer in the presence of 400 mM salt, 0.2 nM signal peptide was added to the cis (periplasmic) side of the membrane with no effect on membrane conductance (Figure 6). However, after addition of 0.2 nM signal peptide to the trans compartment of the same membrane, there were the usual step increases of conductance.

Discussion

Signal Sequence-Gated Channels

We propose that signal sequences are the physiological ligands that open protein-conducting channels. The observation that signal peptides open large aqueous channels in E. coli plasma membrane both corroborates and enlarges upon our previous observation that similar conductances are observed in mammalian ER upon release of nascent translocating chains (Simon and Blobel, 1991). The demonstration that two channels have similar conductance does not mean that they are identical. However, the observation that a large conductance is observed with manipulations of either the initiation or termination of chain translocation provides a compelling argument for the existence of protein-conducting channels.

The current report demonstrates that the opening of the channel is coupled to the initiation of translocation; specifically, presentation of the signal sequence to the channel. This is an essential, perhaps the essential, function for the signal sequence. If opening of the channels were not tightly coupled to the initiation of translocation, then a sewer pipe would be open between the cytosol and the periplasmic space, a conductance large enough to kill the cell.

Clearly both exposure of the channel to signal peptides and opening of the channel to the flow of ions represent a nonphysiological situation. However, there are cogent reasons to believe that these results reflect the in situ physiology. Our results reveal that at physiological voltages, the plasma membrane of E. coli is very much like mammalian plasma membranes, extremely impermeant to the flow of ions. In situ the channel would never be expected to be freely conductive to ions and metabolites. A signal sequence is normally presented in the context of larger protein. When the signal sequence gates the channel open, the adjacent sequence would insert into and thus occlude the channel. We believe that the concentrations of signal peptide used are reasonable, although it is difficult to say what would be a "physiological" amount. In these studies, we observed the openings of discrete individual channels at 0.2 nM signal peptide and a macroscopic conductance increase at 200 nM. There are 10^{12} cells in 1 ml of packed E. coli giving an average volume of 1 fl (10^{-15} liters) per cell (Roberts et al., 1963). Thus, our macroscopic conductance was observed with a signal peptide concentration of 120 peptides per E. coli and individual channel openings at a concentration of ~1 peptide per 8 cells. In contrast, the concentrations of signal peptide used to perturb bilayer structure, 100 μM (Killian et al., 1990), correspond to 60,000 signal peptides per E. coli.

Signal Peptides Bind within the Channel

The effects of high salt on the gating of the channel, and especially on the voltage dependence, suggest that the signal peptide is binding within the mouth of the pore. Higher than physiological concentrations of salt had two effects on the signal peptide–gated conductance. First, when the channels opened, they stayed open, whereas at low salt they flickered open and closed. High salt concentrations reinforce hydrophobic bonds. All signal sequences have a short stretch of hydrophobic amino acids. If this stretch is important for binding to the protein-conducting channel, it is expected that this bond would be reinforced at higher salt concentrations. We suggest that at 50 mM salt, the channel opens and closes as the signal peptide reversibly binds and dissociates. At salt concentrations of 600 to 1000 mM, the peptide remains bound, and the channel remains open. A second effect of the high salt was the loss of the channel's sensitivity to membrane potential; the channels remained open at both positive and negative membrane potentials. Under low salt conditions, the channels opened and closed at positive potentials and remained closed at negative potentials. This implies that there is a voltage dependence to the gating of this channel. Like the sodium or calcium channels, it would be closed at negative, physiological potentials and only open at more positive membrane potentials. Alternatively, the voltage could affect the binding of the signal peptide to the channel. All signal peptides are positively charged dipoles. At positive membrane potentials, their local concentration in the channel would be increased. Similar phenomena have been observed for other charged molecules, such as tetraethylammonium, that enter into and bind a channel only when there is an electrical potential difference across the

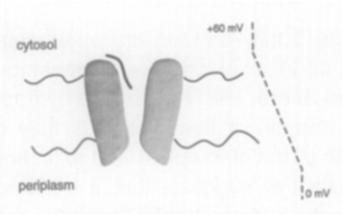

Figure 7. The Signal Peptide Binds in the Mouth of the Channel

This is a schematic for the location of a binding site for the signal sequence within the channel. The gradient of voltage exists only across the membrane, not in the bulk solution. Within the bulk cytoplasmic solution, the voltage will be close to an isopotential +60 mV. Within the bulk periplasmic solution, it will be close to an isopotential 0 (ground). Thus, if voltage affects signal peptide binding, the binding site must be in a region where there is a voltage gradient. The dashed line is a schematic for the voltage. The solid dark line indicates the signal peptide.

membrane. Additionally, the voltage gradient across the membrane may align the signal peptide at the proper orientation in the channel for binding. Thus, we suggest the membrane potential does not directly gate the channel. Instead it indirectly gates the channel by affecting the effective concentration of signal peptide at its binding site. Consistent with this explanation is the observation that at higher salt conditions (which reinforce hydrophobic bonds), negative voltages no longer close the channel.

If indeed the membrane potential affects binding of the signal peptide, then the signal peptide is exposed to the voltage gradient. Thus, it must bind to the channel at a site that is at least partially within the plane of the membrane (Figure 7). This is consistent with the observations that a signal sequence (depending upon where it is located within a protein) can function both as a signal sequence to initiate translocation and as a transmembrane segment (Friedlander and Blobel, 1985). A number of integral membrane proteins have been shown to have internal signal sequences that function as transmembrane domains (Mostov et al., 1981). Additionally, when the amino terminus of the vesicular stomatitis virus glycoprotein signal sequence is elongated, it continues to function as the signal sequence, but instead of being cleaved, it now functions as a transmembrane domain as well (Shaw et al., 1988). These results imply that the signal sequence binds not outside the channel, but further into the mouth at a site from which—if the protein is so encoded—it can subsequently be displaced from the lumen of the channel and partition laterally into the lipid bilayer (see discussion of translocation of integral membrane proteins in Simon and Blobel, 1991).

Other Functions for Signal Sequences

Three other distinct functions have been proposed for the signal sequence. First, it is essential for targeting the nascent chain to the membrane by binding to cytosolic factors (signal recognition particle [SRP] or SecB) (Walter and Blobel, 1981; Watanabe and Blobel, 1989). These cytosolic factors, in turn, mediate targeting by binding to membrane-bound receptors (Gilmore et al., 1982). Second, the signal sequence retards folding and thus keeps the nascent chain in a translocation-competent form (Park et al., 1988). Third, signal sequences, by virtue of their short hydrophobic stretches, initiate translocation by partitioning into the lipid bilayer (Engelman and Steitz, 1981; Von Heijne and Blomberg, 1979).

A compelling body of evidence supports a role for the signal sequence in targeting proteins to membranes. The binding affinity of SRP for a ribosome increases 6000-fold when a protein is being synthesized with a signal sequence (Walter et al., 1981). The signal sequence can be specifically cross-linked to the 54 kd subunit of SRP (Kurzchalia et al., 1986) even at temperatures where molecular motions have been significantly slowed (Krieg et al., 1986). The interaction of SRP with the membrane is a key step in translocation. A membrane-bound SRP receptor has been identified that catalyzes the GTP-dependent displacement of SRP from the signal sequence (Gilmore et al., 1982; Connolly and Gilmore, 1989). Translocation is blocked if this receptor is cleaved with protease and can be restored if the proteolyzed fragment is added back to the membranes (Walter, et al., 1979, Meyer and Dobberstein, 1980). Finally, SRP is required for all mammalian in vitro translocation assays. Two experiments stand in contrast with these results. It has been demonstrated that for short peptides (<60 aa), cytosolic factors such as SRP or SecB are not needed for translocation (Zimmermann et al., 1990; Cobet et al., 1989). Second, deletion of SRP in yeast is not lethal, though it does produce a significant slowing of growth (Hann and Walter, 1991).

Together, these results suggest that there are two consequences of the interaction of the signal sequence with SRP on protein targeting. Protein translation is slowed, keeping the protein in a conformation permissive for translocation. Second, binding of SRP to its receptor facilitates targeting the nascent chain to the membrane, thereby accelerating the translocation process. However, the SRP–SRP receptor can be bypassed.

It has been suggested that the primary function of the signal peptide is not to interact with extrinsic factors, but to keep the mature protein in a translocation-competent state (Wickner, 1980; Randall and Hardy, 1989). Yet, in ribosomes whose translation has been halted by SRP, the signal sequences are specifically, and exclusively, cross-linked to the 54 kd subunit of SRP, demonstrating that signal sequences do interact with other factors (Kurzchalia et al., 1986; Krieg et al., 1986). In mammalian cells, all proteins are translocated as they are being synthesized, and in E. coli most proteins initiate their translocation before they are fully synthesized (Josefsson and Randall, 1983). Indeed signal sequences are often cleaved off before all of a protein is made (Gilmore and Blobel, 1985). These results indicate that the signal sequence is unlikely to be required for keeping the mature portion in a translocation-competent form.

It has been argued that signal sequences are required initially to partition proteins into the lipid bilayer to initiate translocation (Von Heijne and Blomberg, 1979; Engelman and Steitz, 1981; Dierstein and Wickner, 1985). This is based on the observation that one of the few features in common between signal sequences is a stretch of hy-

drophobic amino acids. Consistent with this hypothesis are the observations that at 100 μM, the signal peptides of PhoE and M13 coat protein change the ^{31}P nuclear magnetic resonance signals of lipid structure (Killian et al., 1990). Additionally, when 0.4 mM LamB signal peptide (the same as used in this study) is pressure injected under a monolayer of lipid (final concentration 3 μM), there is a change of the packing pressure in the film, indicating an interaction of the signal peptide with the lipid (Briggs et al., 1986).

There are a number of reasons why we reject the idea that the interaction of signal sequences with lipid is important for translocation. First, signal sequences can compete with nascent translating proteins for translocation. This indicates that there is a distinct binding site for the signal sequence; they are not nonspecifically partitioning into the bilayer. Second, there is no evidence to indicate that the signal sequence is ever directly exposed to the bilayer under physiological circumstances. The signal peptide's first known interaction is with a protein, the 54 kd subunit of SRP. Third, the minimal concentration that disturbed bilayer structure, 100 μM, is the equivalent concentration of 60,000 signal peptides per E. coli and may not represent a physiologically relevant interaction. The results presented in this paper demonstrate that conditions that accentuate hydrophobic interactions keep the signal sequence–gated channel in an open conformation (see Figure 5 and Figure 6). This suggests that the hydrophobic segment of the signal sequence is important for its interaction with its binding site in the protein-conducting channel.

The translocation of proteins through an aqueous channel rather than through the lipid bilayer would help explain a number of observations. First, translocation intermediaries can be extracted from membranes with aqueous perturbants (Gilmore and Blobel, 1985). Second, numerous proteins with hydrophobic stretches are translocated across membranes (Garoff et al., 1978; Frank et al., 1978). If these hydrophobic stretches transiently experienced the hydrophobic milieu of the membrane, then it would cost the cell dearly to extract them on the other side of the membrane. These hydrophobic stretches are initially synthesized in the ribosome and emerge in an aqueous milieu. There would be considerable energy gain if these were to partition into a membrane. However, for the cell to push them along through an aqueous milieu does not cost additional energy. Thus, it makes sense that they are continuously kept in a hydrophilic environment as they traverse the membrane.

These results suggest that such a mechanism has been conserved between the E. coli plasma membrane and mammalian ER. It is tempting to speculate that pores are a more general mechanism for moving proteins across membranes. Some evidence suggests that the mitochondria and chloroplast use a similar mechanism. The vesicular stomatitus virus glycoprotein is normally targeted to the ER, and it has a single transmembrane domain. If its signal sequence is replaced with a signal for the chloroplast, then the protein crosses both the outer and inner chloroplast membrane (Lubben et al., 1987). If it is retargeted to the mitochondria, it fully crosses the outer membrane and ends up as an inner membrane transmembrane protein (Nguyen and Shore, 1987). Thus, the hydrophobic transmembrane domain of this protein is fully translocated across some of the chloroplast and mitochondrial membranes. Again this suggests that a translocating chain never tastes the hydrocarbon milieu of the membranes of these organelles. More likely, the process that stops a protein from translocating across a bilayer does not depend on hydrophobic interactions with the lipid bilayer but specific interactions with the translocation machinery.

The machinery for signal sequence mediated translocation across the ER is clearly biochemically distinct from the ATP-binding cassette proteins that have been characterized in the ER for peptide transport. Cells deficient in the peptide transporters were able to present a peptide to the major histocompatibility complex I in the ER if a signal sequence was placed on the peptide (Anderson et al., 1991). These ATP-binding cassette transporters have been characterized as peptide "pumps" or "flipases." Many of these transport short peptides. However, some of them, such as HlyB, can transport proteins in an unfolded conformation of over 1000 aa (Koronakis et al., 1989, Blight and Holland, 1990; Gilson et al., 1990). The biophysical challenge of moving a long, often hydrophilic, molecule across the membrane is not unlike translocation across the ER or E. coli plasma membrane. Thus, we suspect that these may also function as protein-conducting channels. It has been suggested that ATP is required for "pumping" each amino acid across the bilayer. Alternatively, the ATP may be required only for gating the channel into a permissive state for translocation.

Experimental Procedures

Formation of Planar Bilayers

Planar lipid bilayers were formed across a hole (0.2 to 1.2 mm diameter) in a Teflon partition as previously described (Mueller et al., 1962; Simon et al., 1989; Simon and Blobel, 1991). All lipids were from Avanti-Polar (Birmingham, AL), and the lipid solutions in these experiments were 10 mg/ml diphytanoylphosphatidylcholine, 5 mg/ml phosphatidylethanolamine, and 5 mg/ml phosphatidylserine dissolved in decane (Fluka). Membranes were initially formed in solutions of 50 mM KCl and 5 mM K–HEPES (pH 7.5). After formation of the membrane, an osmotic gradient was formed by addition of aliquots of either 50% glycerol, 8 M urea, or 4 M KCl to the cis side. Each chamber held 4 ml of solution.

Preparations of Membranes

Three different E. coli plasma membrane preparations were fused to the bilayer. InV were prepared as previously described (Müller and Blobel, 1984). The membranes were stored frozen at 10 A280 U/ml. Protoplasts were made as previously described (Birdsell and Cota-Robles, 1967). Outside-out membranes were formed as previously described (Kaback, 1971).

Fusion of Vesicles to Bilayers

Vesicles were fused to the bilayer as previously described (Miller and Racker, 1976; Zimmerberg et al., 1980; Cohen et al., 1989; Simon et al., 1989; Simon and Blobel, 1991). After formation of the bilayer, an osmotic gradient was established by the addition of either 50% glycerol, 8 M urea, or 4 M KCl (each to a final concentration of 300 mM) to the cis chamber. Vesicles were loaded into a small glass micropipet with the mouth diameter of ~20 μm. The pipet was brought up to the cis side of the bilayer, sometimes touching the bilayer, and the vesicles

were pressure ejected while the solutions in both chambers were vigorously stirred. If fusion was not observed immediately, the osmotic gradient was increased, sometimes as high as 1 M osmoticant. After fusion, the osmotic gradient was eliminated by added osmoticant to the trans chamber or by perfusing out the osmoticant from the cis chamber.

Electrophysiology

All electrophysiological techniques were as previously described. However, for some experiments, a commercial voltage clamp (Axopatch 200, Axon Instruments, Foster City, CA) was used instead of the homemade electronics previously described. All current records are plotted as recorded. The conductance is plotted at the current divided by the applied voltage. All capacitive transients and mechanical artifacts are left intact.

Reagents

The signal peptide used in these experiments was the cleavable signal sequence of the LamB protein (Emr and Silhavy 1983). Its sequence is: MMITLRKLPLAVAVAAGVMSAQAMA. It was synthesized by the Rockefeller University protein synthesizing facility. The purification was checked by high pressure liquid chromatography.

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