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The translocation of negatively charged residues across the membrane is driven by the electrochemical potential: evidence for an electrophoresis-like membrane transfer mechanism

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The role of the membrane electrochemical potential in the translocation of acidic and basic residues across the membrane was investigated with the M13 procoat protein, which has a short periplasmic loop, and leader peptidase, which has an extended periplasmically located N-terminal tail. For both proteins we find that the membrane potential promotes membrane transfer only when negatively charged residues are present within the translocated domain. When these residues are substituted by uncharged amino acids, the proteins insert into the membrane independently of the potential. In contrast, when a positively charged residue is present within the N-terminal tail of leader peptidase, the potential impedes translocation of the tail domain. However, an impediment was not observed in the case of the procoat protein, where positively charged residues in the central loop are translocated even in the presence of the membrane potential. Intriguingly, several of the negatively charged procoat proteins required the SecA and SecY proteins for optimal translocation. The studies reported here provide insights into the role of the potential in membrane protein assembly and suggest that electrophoresis can play an important role in controlling membrane topology.

Key words: membrane insertion/membrane potential/protein translocation

Introduction

During recent years, a number of laboratories have investigated what controls the membrane topology of proteins (Boyd and Beckwith, 1989; Laws and Dalbey, 1989; von Heijne, 1989; Nilsson and von Heijne, 1990; McGovern *et al.*, 1991; for review see Boyd and Beckwith, 1990; Dalbey, 1990; von Heijne, 1994) and this effort has led to the rules that govern membrane topology. The 'positive inside' rule (von Heijne, 1986; von Heijne and Gavel, 1988) states that basic residues bordering transmembrane segments direct the membrane insertion and orientation of membrane proteins. It is now possible to use this 'positive inside' rule and hydrophobicity analysis to correctly predict 95% of the membrane topologies of bacterial membrane proteins (von Heijne, 1992).

While the 'positive inside' rule is well established, it is not clear how positively charged residues exert their effects on topology and are retained in the cytosol. Since an electrochemical potential is required for the translocation of many membrane and secreted proteins (Date *et al.*, 1980; Daniels *et al.*, 1981; Enequist *et al.*, 1981), one possibility is that the topological effects of basic residues are due to the potential which renders the periplasmic side of the membrane positively charged. This would impede the transfer of positively charged residues and may be the basis of the 'positive inside' rule. Likewise, the membrane potential may promote translocation of negatively charged residues across the membrane. Electrophoretic effects have recently been described for mutant pro-OmpA proteins (Geller *et al.*, 1993), as well as for certain mutant leader peptidase proteins (Anderrson and von Heijne, 1994). However, in some cases, electrophoresis may play only a minor role, since uncharged and even positively charged regions were found translocated (Bakker and Randall, 1984; Kuhn *et al.*, 1990; Kato *et al.*, 1992).

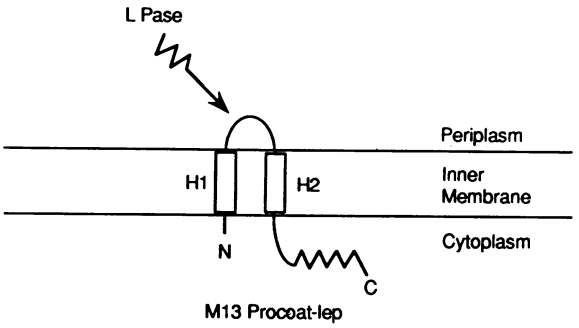
In this paper we have examined the role of the potential in the translocation of negatively and positively charged residues across the membrane. Two proteins were studied, namely the M13 procoat protein and leader peptidase. For analytical reasons both proteins were modified. M13 procoat protein carried a C-terminal extension with an antigenic tag, whereas leader peptidase was modified by extending the N-terminal tail. We find that the potential promotes the translocation of negatively charged residues within the central loop of the M13 procoat protein, as well as within the N-terminal tail of mutant leader peptidase proteins. In addition, we find that the potential impedes N-terminal translocation when a basic residue is present in the tail. These results suggest that the potential may act electrophoretically and, in this fashion, may help to control the membrane topology of proteins.

Results

The role of the membrane potential in transferring negatively charged residues

We have investigated the role of the potential in the translocation of charged residues within the central loop of the M13 phage procoat protein and within the N-terminal tail of leader peptidase proteins. The M13 procoat protein is synthesized with a typical leader peptide of 23 amino acid residues and its insertion into the membrane occurs as a loop involving the two hydrophobic domains (Kuhn, 1987) and requires an electrochemical membrane potential (Date *et al.*, 1980), but not the SecA or SecY protein (Wolfe *et al.*, 1985). After translocation of the central region across the membrane, procoat protein is cleaved by leader peptidase to generate a bitopic membrane

A



B

Pc-lep Constructs	H1 AEGDDPAKAAFNSLQ... H2	Net Charge	Processing % − CCCP	Processing % + CCCP
1. 0PC-lep	AQGNN...	0	99	97
2. -1PC-lep	AQGND...	-1	95	95
3. -2PC-lep	AQGDD...	-2	94	87
4. -3PC-lep (W.T.)	AEGDD...	-3	91	58
5. -4PC-lep	AEEGDD...	-4	99	0
6. -5PC-lep	AEEEGDD...	-5	99	0
7. -6PC-lep	AEEEEGDD...	-6	62	0
8. -7PC-lep	AEEEEEGDD...	-7	0	0

Fig. 1. (**A**) Membrane topology of procoat−lep. Apolar domains are represented by rectangles; the lep domain by a zigzag line. (**B**) Procoat−lep constructs with negatively charged amino acids introduced immediately after H1 and their processing properties. The net charge in the periplasmic loop region and the percent processing for each mutant is shown. The percent processing was determined as described in Materials and methods.

protein with one transmembrane segment that is oriented with its C-terminus in the cytosol. To determine whether the membrane potential promotes transfer of negatively charged residues across the membrane, we used oligonucleotide-directed mutagenesis (Zoller and Smith, 1983) to make mutations within the short periplasmic loop of the M13 procoat protein. The mutations that were created and the resulting net charge immediately after the leader peptide of each mutant are shown in Figure 1B. The procoat mutants were cloned into a plasmid where their expression is arabinose-inducible (Kuhn and Wickner, 1985). In these studies, the procoat protein also contains a leader peptidase fragment of 103 residues fused to the C-terminal region of the protein (Figure 1A, lep is depicted by zigzag line), which allows the proteins to be immunoprecipitated with an anti-leader peptidase antibody. It was necessary to add this lep fragment because anti-procoat protein antibody was ineffective in precipitating many of the mutant proteins. Previously we showed that this fragment does not disturb the membrane assembly kinetics of procoat protein (Kuhn *et al.*, 1986).

Cells synthesizing the procoat−lep protein with a net charge in the periplasmic loop that ranges from 0 to −7 were pulse-labeled with ^{35}S-translabel for 1 min and chased for 5 s with non-radioactive methionine. Cells were then analyzed for processing by immunoprecipitation with antibody to the C-terminal lep fragment, followed by SDS−PAGE and fluorography. As shown in Figure 2A, procoat protein with a net charge after the leader peptide of 0 to −5 was efficiently processed when a membrane potential was present (no added carbonyl cyanide *m*-chlorophenylhydrazone; CCCP), indicating that it had assembled across the membrane and was cleaved by leader peptidase. In contrast, processing of procoat protein was slowed or blocked as the number of negatively charged residues were increased further after the leader peptide. Procoat protein with a net charge in the loop of −6 was only 62% processed in the 1 min pulse-label, while procoat protein with a net charge of −7 was not processed at all.

We next asked whether the membrane electrochemical potential is required for membrane insertion of these procoat protein mutants. To test this, the membrane potential was collapsed, prior to pulse-labeling the cells, by the addition of CCCP, a protonophore. Cells (1 ml cultures) were pre-treated for 45 s with 5 μl 10 mM CCCP, labeled with ^{35}S-translabel for 1 min, chased with non-radioactive methionine for 5 s and analyzed for cleavage as described above. In these studies we also confirmed that the precursor to the outer membrane protein A (pro-OmpA), which requires the membrane potential for export, accumulates in cells pretreated with CCCP. Figure 2A shows that as the number of negatively charged residues are progressively increased, processing becomes more and more dependent on the membrane potential

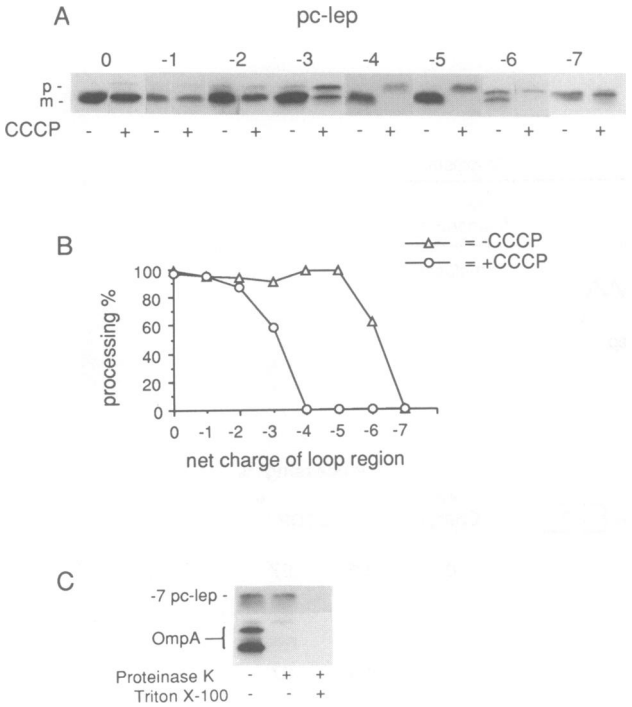


Fig. 2. (**A**) Processing of procoat−lep proteins with or without CCCP treatment. MC1061 cells bearing plasmids encoding procoat−lep proteins were grown to mid-log phase, induced with 0.2% arabinose for 5 min and then labeled with 20 μCi ^{35}S-translabel for 1 min and chased for 5 s with non-radioactive methionine. Proteins were acid precipitated and subsequently immunoprecipitated with anti-leader peptidase antiserum and analyzed by SDS−PAGE and fluorograghy. Where appropriate, treatment with CCCP was carried out after 5 min of induction by arabinose. CCCP (5 μl, 10 mM) was added to a 1 ml cell culture and, after 45 s, cells were labeled with ^{35}S-translabel and analyzed exactly the same as for the no CCCP treatment samples. p, the precursor form; m, the mature form. (**B**) Processing becomes more membrane potential-dependent as the number of negatively charged residues in the loop region is increased. Translocation was determined by the percent processing (see Materials and methods). (**C**) Protease mapping studies of the −7 procoat−lep construct. Cultures (1 ml) of MC1061 cells transformed with the plasmid encoding the −7 procoat−lep protein were grown to mid-log phase, induced with 0.2% arabinose for 5 min and then labeled with 100 μCi ^{35}S-translabel for 1 min. Cells were then collected and converted into spheroplasts by lysozyme treatment, as described in Materials and methods. The spheroplasts were incubated with or without proteinase K (final concentration 1 mg/ml) on ice for 1 h. A lysis control was included by adding proteinase K (1 mg/ml final concentration) and Triton X-100 (2% final concentration). Samples were then immunoprecipitated with antiserum to leader peptidase and OmpA and analyzed by SDS−PAGE and fluorography.

(compare + and −CCCP lanes). Cells expressing procoat proteins with a −1 or −2 net charge in the loop were processed to nearly the same extent without a potential, whereas the wild-type protein (−3PC−lep) with a −3 net charge was processed to only 58% of the level with the potential present. In contrast, processing was completely dependent on a membrane potential when the net charge in the loop domain was −4 to −6. Quantitation of the percent processing, as shown in Figure 2B, clearly indicates that a membrane potential is an absolute requirement for the procoat protein mutants with a loop net charge of from −4 to −6.

Protease mapping was performed to show that the −7 procoat protein mutant, which was not processed by leader peptidase, was indeed not inserted across the membrane. Cells synthesizing procoat−lep with a −7 net charge (in the loop) were pulse-labeled with ^{35}S-translabel for 1 min, converted to spheroplasts and then treated with 1 mg/ml proteinase K for 60 min. As a control, Triton X-100 (2% final concentration) was added to one sample to break open the cells. After the proteinase K was quenched with phenylmethylsulfonylfluoride (PMSF), the samples were immunoprecipitated with antiserum to leader peptidase and OmpA. Figure 2C shows that the −7 procoat−lep protein was resistant to proteinase K under conditions where OmpA, the positive control, was digested by the protease. This result confirms that the −7 mutant protein did not insert across the membrane.

To determine whether there is a positional dependence of the inserted negatively charged residues, we examined other mutant procoat−lep proteins in which the acidic residues were introduced further away from the leader peptide (Figure 3). Membrane potential-dependent insertion was observed for the procoat protein mutants that have a net charge of −3 and −4 after the leader peptide. This is illustrated in Figure 4, where processing was analyzed in an identical fashion to that described in the legend to Figure 2. With a potential, procoat protein with a −3 net charge was processed efficiently (Figure 4, −3M), whereas processing was slower for the −4 mutant (Figure 4, −4M). In contrast, only very little processing was observed for the −5 mutant, even with a potential (Figure 4, −5M). As shown in the protease mapping study in Figure 4B, the majority of the procoat protein −5 mutant is not accessible to proteinase K added to spheroplasts, confirming that the −5 mutant protein cannot insert across the membrane.

As was observed for the mutants with negative charges introduced immediately after the leader peptide, we find a membrane potential dependence for mutants with charged residues introduced into the middle of the periplasmic loop domain. In CCCP-treated cells, processing of procoat protein with a −3 (−3M), −4 (−4M) or −5 (−5M) net charge in the loop was completely blocked. It seems, by comparing the two sets of mutants (Figures 2 and 4), that the negatively charged residues have more severe effects on translocation when introduced into the middle of the loop than immediately after the leader peptide. However, this may be due to the fact that the loop size is larger by three amino acid residues for the M constructs with the same net charge. Nevertheless, in both cases, potential-dependent translocation is observed when the net charge in the region exceeds −3.

We next tested whether a potential is required for the translocation of N-terminal tails of membrane proteins when they contain acidic amino acid residues by investigating various leader peptidase mutant proteins (Figure 5). Previously we showed that a membrane potential is required for insertion of the N-terminal tail across the membrane of a leader peptidase fusion protein (termed 38 Pf3−leader peptidase R79) comprising the first 18 residues of the Pf3 bacteriophage coat protein and 20 neutral residues fused to the N-terminus of leader peptidase. In this protein the phenylalanine at position 79 of leader peptidase was mutated into an arginine residue such that the only domain that moves across the membrane (Cao and Dalbey, 1994) is the N-terminal tail (Figure 5A), in contrast to the wild-type leader peptidase that spans the

Pc-lep Constructs		Net Charge	Processing % − CCCP	+ CCCP
1. 0PC-lep	H1 AQGNNPAKAAFNSLQASATE H2	0	99	97
2. -3MPC-lep	AQGNNPAKAAEDDFNS...	-3	98	0
3. -4MPC-lep	AQGNNPAKAAEEDDFNS...	-4	46	0
4. -5MPC-lep	AQGNNPAKAAEEEDDFNS...	-5	18	2

Fig. 3. Mutants of procoat−lep with negatively charged amino acids inserted into the middle of the loop region and their processing properties. M indicates that the negatively charged amino acids are in the middle of the loop region. Processing was determined as described in Materials and methods.

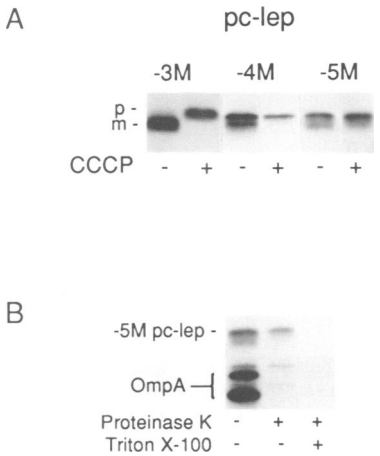


Fig. 4. (**A**) The role of the potential in the processing of procoat−lep mutants with negatively charged residues inserted into the middle of the loop region. MC1061 cells expressing procoat−lep −3M, −4M and −5M respectively were analyzed as described in the legend to Figure 2A. (**B**) Protease mapping of the −5M procoat−lep protein. MC1061 cells expressing the −5M procoat−lep protein were labeled and analyzed as described in the legend to Figure 2C.

membrane twice with a large C-terminal domain (P2) in the periplasm (Wolfe *et al.*, 1983; Moore and Miura, 1987; Lee *et al.*, 1992). Sec-dependent translocation of the large P2 domain of leader peptidase is prevented in Pf3−leader peptidase (Cao and Dalbey, 1994) and wild-type leader peptidase (Zhu and Dalbey, 1989) by inserting the arginine at position 79. These and other studies (Lee *et al.*, 1992) demonstrate that insertion of the H1 domain of the protein occurs independently of insertion of the H2 domain.

The mutants described in Figure 5 are named with the number at the left of lep indicating the charges within the tail region, whereas numbers to the right of lep refer to the length of the tail. In addition, the C designates that the charges are placed in the C-terminal region of the tail and N indicates that the charges are located near the N-terminus of the tail. Translocation of the N-terminal tails studied here was membrane potential-dependent only when negatively charged residues are located in the tail domain (Figure 5B). Leader peptidase (−2lep38) with a 38 residue tail contains two aspartic acid residues within its N-terminal tail, at positions 7 and 18. Previously we showed that this mutant inserts only in the presence of a membrane potential (Cao and Dalbey, 1994). Here we tested whether a potential is also required for translocation of this region when these two acidic residues were mutated to neutral residues. Exponentially growing cells expressing 0lep38, lacking the negatively charged residues, were pre-treated with CCCP in order to collapse the membrane electrochemical potential. Cells were then labeled with ^{35}S-translabel for 1 min and converted to spheroplasts in order to test for N-terminal translocation using the protease mapping procedure (Cao and Dalbey, 1994) and analyzed using this procedure as before. Aliquots were immunoprecipitated with antiserum to leader peptidase (lep), OmpA and ribulokinase (araB). We confirmed that OmpA, which is only exposed on the periplasmic surface of the outer membrane, is fully digested, while AraB, a cytosolic protein, remained inaccessible to digestion (data not shown). These two controls demonstrate that the inner membrane remained intact and the outer membrane was permeabilized, during proteinase digestion. Figure 6A shows that, in the absence of a membrane potential, the uncharged 38 residue tail was efficiently translocated. The protease-protected fragment, which lacks the tail region, runs at the same position as the undigested wild-type leader peptidase (data not shown). These data are consistent with the potential being required only to promote translocation of acidic residues. We also examined whether the potential is required for translocation of a 23 residue tail with only one negatively charged residue (immediately before the transmembrane segment). Previously we had observed that the 23 residue tail containing no charged residues can be efficiently translocated in the absence of a potential (Cao and Dalbey, 1994). Oligonucleotide-directed mutagenesis was used to substitute the proline residue before H1 with a glutamic acid residue. The −1Clep23 protein with one negatively charged residue in the tail was translocated in the presence of a potential, as indicated by the generation of the protected band (Figure 6B, arrow). It can also be partially translocated across the membrane in the absence of a potential, although a potential is required for efficient translocation. The −2Clep 23, with two glutamic acid residues introduced in the tail, was efficiently translocated in the presence of a potential (Figure 6C, −CCCP), but not without a potential (Figure 6C, +CCCP). We also examined the potential requirement with another mutant with four negatively charged residues located in the N-terminal end of the tail region. This mutant protein inserted (Figure 6D, arrow), albeit inefficiently, into the membrane only in the presence of a potential (Figure 6D; compare −CCCP with +CCCP results). Thus, these results demon-

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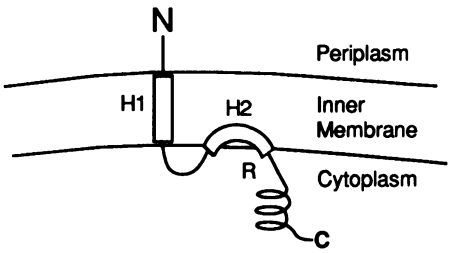

B

Lep Constructs	Charge(s) within the Tail	Translocation − CCCP	Translocation + CCCP
1.* -2lep38	-2	+	−
2. 0lep38	0	+	+
3.* 0lep23	0	+	+
4. -1Clep23	-1	+	+/−
5. -2Clep23	-2	+	−
6. -4Nlep23	-4	+/−	−

Fig. 5. (**A**) Membrane topology of Pf3−lep and its derivatives. Apolar regions H1 and H2 are represented by rectangles and the helix represents the H3 and the P2 domains of leader peptidase. R indicates that an arginine is present after H2 to prevent translocation of the P2 domain; this simplifies the protease mapping, as only the N-terminus can be translocated (Cao and Dalbey, 1994). (**B**) Mutants of leader peptidase with extended tails and their membrane translocation properties. Numbers on the left of lep indicate the net charge within the tail region, while numbers to the right of lep refer to the length of the tail. C and N indicate whether the charges are near the C-terminus of the tail (C) or near the amino terminus (N). +, >85% translocation; −, <15% translocation; +/−, between 15 and 85% translocation. Constructs 1 and 3, which are highlighted by the *, were analyzed by Cao and Dalbey (1994).

strate that the membrane potential can only promote the transfer of a limited number of acidic residues within the N-terminal tail of proteins.

The role of membrane potential in the translocation of positively charged residues

If the membrane potential (periplasmic side positive) is acting electrophoretically on translocated regions of the substrate, it should impede the transfer of positively charged residues. We tested this using leader peptidase and procoat protein constructs into which we had introduced positively charged residues (Figure 7). Starting with a leader peptidase with a 23 residue tail (23 Pf3−lep R79 protein; Cao and Dalbey, 1994), we introduced one positively charged residue at the C-terminal end of the tail by substituting a proline immediately before H1 with an arginine residue. Cells synthesizing +1Clep23 were pulse-labeled with ^{35}S-translabel for 1 min and analyzed for translocation of the tail by the protease mapping technique (Figure 8A). Translocation of the 23 residue tail was blocked when the tail contained a single positively charged residue. However, addition of CCCP to abolish the membrane potential partially restored translocation of the tail. Figure 8A shows that, in CCCP-treated cells, 58% of this protein was accessible to protease and converted to a shorter form (see arrow). This was also observed, although to a decreased extent, with a leader peptidase protein (+1Mlep 23) with an arginine residue substituted for the glycine at position 13 within the tail. The N-terminal tail could be only partially translocated in cells treated with CCCP (Figure 8A, +1Mlep23). The introduction of two positively charged residues into the tail by the addition of a lysine at position 22 and an arginine at position 23 abolished translocation with or without a membrane potential (Figure 8A, +2Clep23). Thus, even in the absence of a potential, positively charged residues have an inhibitory effect on membrane translocation of the polypeptide chain.

Previously we showed that positively charged residues located within the short periplasmic loop of the M13 procoat protein can be translocated across the membrane in the presence of a potential (Kuhn *et al.*, 1990). Given our membrane potential results with basic residues in the N-terminal tail of leader peptidase, we re-examined some of the positively charged mutants of the procoat protein. Procoat−lep ARGNN, with a +1 net charge in the periplasmic loop, was processed in a 1 min pulse-label with ^{35}S-translabel (Figures 7B and 8B). The addition of CCCP, which abolished the potential, as demonstrated by accumulation of pro-OmpA (data not shown), did not stimulate the processing of procoat−lep ARGNN (+1 mutant), indicating that insertion was not promoted. Rather, translocation of the positively charged procoat protein mutants was more efficient in the presence of a

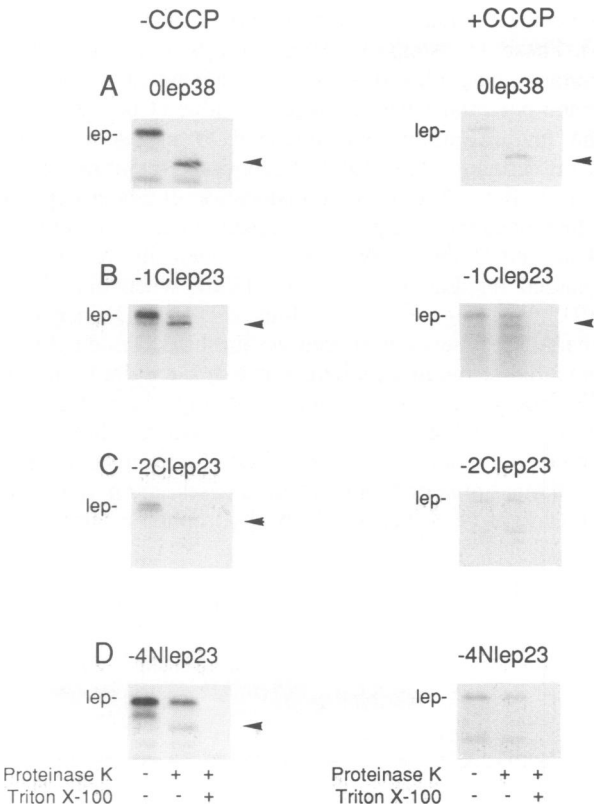

Fig. 6. The role of the potential in translocation of the N-terminal tail. Protease mapping of the Pf3−lep derivatives depicted in Figure 5. Protease mapping studies were performed as described in the legend to Figure 2C. Cells were grown, labeled and analyzed for translocation in the absence of CCCP as described in the legend to Figure 2C. Samples were immunoprecipitated using antiserum to leader peptidase and OmpA. For the CCCP studies, cultures (1 ml) were pre-treated with 5 μl of CCCP (10 mM) for 45 s and then labeled with 100 μCi ^{35}S-translabel as for the mock-treated cells.

membrane potential. We observed this also for procoat−lep ARGRR, with a net charge of +3 in the translocated region. MC1061 expressing procoat−lep ARGRR was processed to the same extent in cells with or without CCCP (+3 mutant; Figures 7B and 8B).

Sec-dependent insertion of procoat proteins with short translocated regions

We checked the Sec-dependency of membrane insertion of a number of the procoat protein mutants to determine whether they insert in a Sec-independent manner, like the wild-type procoat protein (Wolfe *et al.*, 1985). First, we examined whether insertion is SecA-dependent by treating cells with 3 mM sodium azide prior to pulse-labeling; azide treatment specifically blocks SecA function (Oliver, *et al.*, 1990). Procoat proteins with a net charge of −1, −2 or −3 (data not shown), as well as −4 (Figure 9A, left side), inserted normally across the membrane under conditions where translocation of OmpA was inhibited (data not shown). Intriguingly, translocation of procoat with a net charge in the periplasmic loop of −3 (−3M), −4 (−4M), −5 or −6 was inhibited by azide pre-treatment (Figure 9A). These data suggest that SecA is required for optimal insertion of the mutants. We next examined translocation in SecA^{ts} and SecY^{ts} hosts, where the function of the SecA and SecY proteins is impaired at 42°C. Figure 9B and C shows that at the non-permissive temperature processing is measurably impaired for procoat protein −5 but only slightly impaired for the −3M mutant, indicating that these proteins require the SecA/SecY system for optimal translocation. As a control, we showed that processing of the −4 mutant (Figure 9B and C) is unaffected at the non-permissive temperature in these temperature-sensitive strains.

A

Lep Constructs		Net Charge	Translocation - CCCP	Translocation + CCCP
1. +1Clep23	+ H1 lep	+1	-	+/-
2. +1Mlep23	+ H1 lep	+1	-	+/-
3. +2Clep23	++ H1 lep	+2	-	-

B

Pc-lep Constructs		Net Charge	Processing % - CCCP	Processing % + CCCP
1. 0PC-lep	H1 AQGNNDAKAAFNSLQ... H2	0	99	97
2. +1PC-lep	ARGNN...	+1	99	72
3. +3PC-lep	ARGRR...	+3	32	24

Fig. 7. (**A**) Mutants of leader peptidase with positively charged residues within the tail region and their translocation properties. Lep constructs are named as in the legend to Figure 5B. M indicates that the positively charged amino acid is in the center of the tail region. (**B**) Mutants of procoat−lep with positively charged amino acids after H1 and their processing properties.

Discussion

Translocation of protein regions across the membrane occurs either as loops with two hydrophobic flanking regions or as tails with only one flanking hydrophobic region. We have investigated these two insertion modes using the M13 procoat protein and a mutant of leader peptidase that has an extended N-terminal tail and only one adjacent transmembrane domain. For both of these proteins we found that an electrochemical potential is required to transfer negatively charged residues across the membrane. This suggests that the charged residues within a protein are transported across the membrane in an ionic state and that electrophoresis is a driving force in this process.

The membrane potential across the inner membrane in *Escherichia coli* is such that the positive charge is on the periplasmic surface. Therefore, the potential would support membrane transfer of negatively charged residues and restrict transfer of positively charged ones. Indeed, in our experiments this is the case, strongly suggesting that an electrophoretic mechanism does play an important role. First, the membrane potential becomes more progressively involved in membrane assembly of the M13 procoat protein as the number of negatively charged residues in the central domain of the protein increased (Figures 2 and 4). These data support earlier findings on procoat protein mutants, where insertion was less dependent on the membrane potential when the acidic residue at position +2 in the mature region was changed to a leucine residue (Zimmermann *et al.*, 1982). Second, the membrane potential promotes N-terminal translocation of leader peptidase when negative charges are present in a 38 residue tail (Cao and Dalbey, 1994), but not when this region lacks charged residues (Figure 6A). Third, when one (Figure 6B), two (Figure 6C) or four (Figure 6D) negatively charged residues are located within the 23 residue tail the potential is required, whereas it is not required when the 23 residue tail contains no negatively charged residues (Cao and Dalbey, 1994). This demonstrates that a membrane potential is not required for translocation of uncharged tails or loops. In these cases, membrane insertion might be driven solely by hydrophobic interactions.

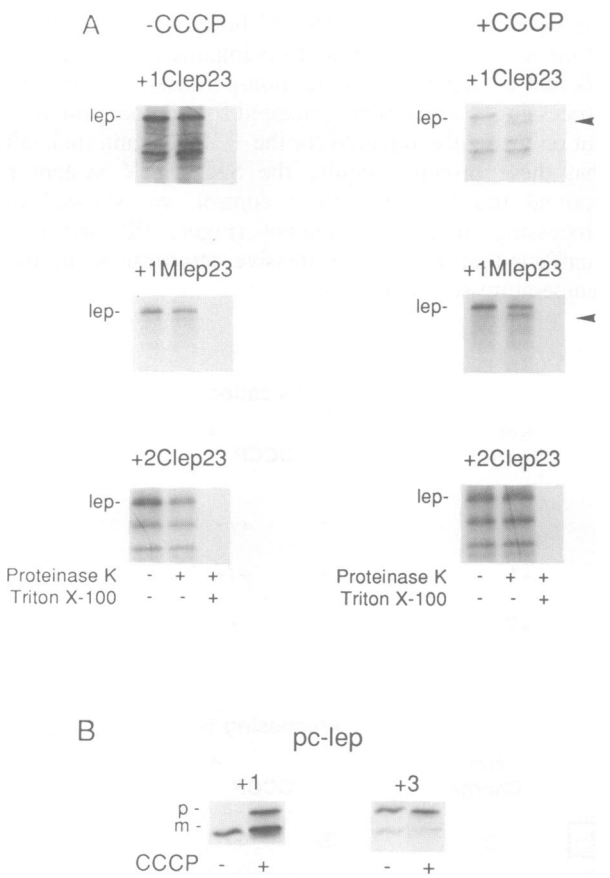

Fig. 8. (**A**) Protease mapping of Pf3−lep derivatives with positively charged amino acids in the tail region. MC1061 cells with plasmids expressing the mutant proteins depicted in Figure 7 were analyzed as described in the legend to Figure 6. (**B**) Processing of procoat−lep ARGNN (+1) and ARGRR (+3) proteins in the presence or absence of a membrane potential. Cells bearing plasmids encoding these proteins were labeled with 20 μCi ^{35}S-translabel and analyzed with or without CCCP as described in the legend to Figure 2A.

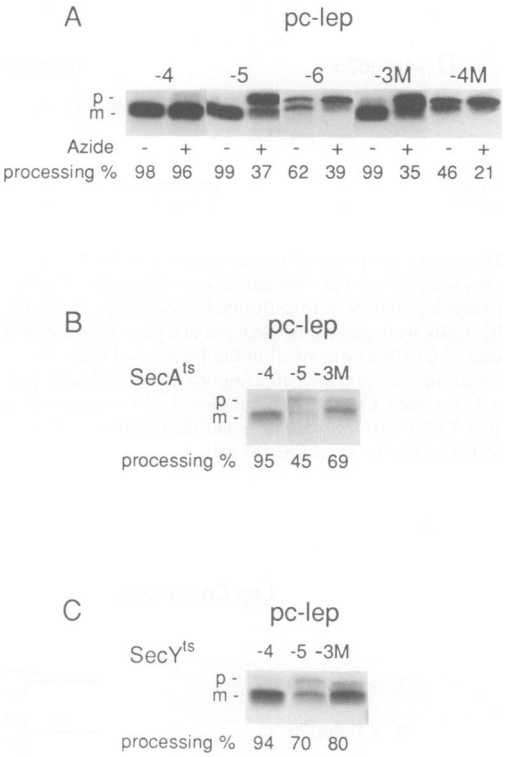

Fig. 9. (**A**) Azide-sensitive translocation of certain procoat−lep mutants with negatively charged residues. MC1061 cells (1 ml) expressing the indicated protein were grown to mid-log phase, induced with 0.2% arabinose for 5 min, treated with 30 μl of sodium azide (100 mM) for 1 min and subsequently labeled with 20 μCi ^{35}S-translabel for 1 min. Samples were acid precipitated and immunoprecipitated with anti-leader peptidase and anti-OmpA antisera and subjected to SDS−PAGE and fluorography. (**B**) Processing of procoat mutants with negatively charged residues at the non-permissive temperature in SecA^{ts} strain (CJ105). CJ105 cells (1 ml) bearing the plasmid encoding the mutant proteins were grown to mid-log phase at 30°C and then shifted to the non-permissive temperature (42°C) for 1 h. Arabinose (0.2% final concentration) was added to induce expression of the plasmid-encoded proteins. After 30 min of induction, cultures (1 ml) were pulse-labeled with 100 μCi ^{35}S-translabel for 1 min and analyzed by immunoprecipitation, SDS−PAGE and fluorography. (**C**) Processing at 42°C of procoat protein mutants in a SecY^{ts} strain (CJ107). CJ107 cells expressing the mutant procoat proteins were grown, labeled and analyzed as described in (B).

Finally, we found that for the N-terminal tail of leader peptidase containing a positively charged residue at two different positions, translocation is prevented by the potential, as it was only translocated when the potential had been destroyed (Figure 8A).

While electrophoretic effects occur for translocation of positively charged residues within the N-terminal tail of leader peptidase, they did not occur within procoat protein (Figure 8B). When the translocated region had a net charge of +1 or +3, the M13 procoat protein inserts in the presence or absence of a membrane potential, with translocation being slower for the +3 mutant. This is not consistent with a simple electrophoretic model and demonstrates that electrophoresis is not the driving force for, at least, these procoat protein mutants (also see Kuhn *et al.*, 1990) and suggests that the inhibitory effects of positive charges are due to other factors (discussed below). In the case of the M13 procoat protein, which is flanked by two hydrophobic regions, the membrane potential might contribute to only a minor extent, because the insertion of procoat protein into the membrane is mainly driven by hydrophobic interactions (Kuhn *et al.*, 1987). This is different from the case of leader peptidase, in which only one hydrophobic domain initiates translocation of the N-terminal tail (Lee *et al.*, 1992).

Other puzzles that are not explained by an electrophoretic mechanism are the following observations. (i) Why does the potential still promote translocation of a model secretory protein that contains no charged residues in the mature domain (Kato *et al.*, 1992)? (ii) Why is ΔpH sufficient to promote export of β-lactamase across the membrane (Bakker and Randall, 1984)? Experiments to address these questions are currently being pursued in our laboratories.

The present data show that the inhibitory effects that charged residues have on membrane translocation are only partly due to the potential and that there must be other factors, such as electrostatic interactions (Gallusser and Kuhn, 1990) or the difficulty of burying charged residues within the bilayer (Summers and Knowles, 1989; Summers *et al.*, 1989), that could hinder translocation. First, in the presence of a potential we find that translocation is blocked when seven negatively charged residues are introduced into the central domain of procoat protein or that translocation is hindered when four negatively charged residues are introduced into the N-terminal tail of leader peptidase. Second, in the absence of a potential we find that translocation is blocked when two basic residues are introduced into the N-terminal tail of a mutant leader peptidase (Figure 8A). This shows that translocation is prevented by charged residues even under conditions where, according to the electrophoretic model, the membrane potential is expected to be optimal for translocation. Third, we found that procoat protein with a +1 or +3 net charge in the periplasmic loop is inserted as efficiently with or without a membrane potential. On the basis of these data, we conclude that the membrane electrochemical potential is not solely responsible for the topological determinants of positively charged residues within membrane proteins.

It is striking that in the absence of a potential, the translocation effects of negatively charged residues are, on balance, similar to those of positively charged residues. This supports the proposal of Engelman and Steitz (1981) that the hydration of charged groups within membrane proteins affects membrane insertion of the peptide chain. For example, procoat proteins with a net charge of −1 or +1 insert across the membrane efficiently (95% for the mutant with a −1 net charge, 72% for the +1 net charge mutant), whereas insertion is, in general, poor with a net charge of −3 or +3 (58% for the wild-type procoat protein with a net charge of −3 and 0% for the 3M mutant with a −3 net charge and 24% for the mutant with a +3 net charge). Similar results were found for translocation of the N-terminal tail of leader peptidase without a potential. Leader peptidase can be partially inserted with a tail region that contains a net charge of −1 or +1, while it cannot be translocated with a tail with a net charge of −2 or +2. In the presence of a membrane potential, the negatively and positively charged residues behave quite differently. One explanation for this might be that with the potential, electrostatic interactions between the membrane surface and negatively charged regions of the membrane protein are weakened, whereas the interactions with positively charged regions are enhanced.

We reported recently that the electrical component of the membrane potential stimulated the rate of membrane insertion of a Sec-dependent pro-OmpA mutant protein with negatively charged residues after the leader peptide and impeded insertion of a pro-OmpA derivative with positively charged residues introduced (Geller *et al.*, 1993). These findings suggested that the membrane electrical potential has a direct effect on translocation of the exported protein and provides the driving force for moving acidic residues across the membrane. Similar findings have been reported by Andersson and von Heijne (1994), who studied the effect of the potential in promoting the membrane insertion of 'inverted' leader peptidase proteins containing negatively charged residues. Intriguingly, by studying a mutant leader peptidase construct with positively charged residues at the very N-terminus of the protein, they found that addition of CCCP resulted in a reversal of the topology from that of an 'inverted' to a 'wild-type' orientation. Although translocation of the N-terminus was not monitored in these studies, they suggested that, in the absence of a potential, the N-terminus containing the positively charged residues was transferred across the membrane, resulting in the reorientation ($N_{peri}C_{cyto}$) of the first hydrophobic region (H1), which causes the topology switch. Indeed, this conclusion is supported by our data, showing that a positively charged residue in the N-terminal tail of leader peptidase can be transferred across the membrane in the absence, but not in the presence, of a potential (Figure 8). In our case, translocation of two positively charged residues did not occur even in the absence of a potential.

Since it is well known that charged residues in the early mature regions of Sec-dependent proteins affect translocation and possibly interaction of the exported protein with the 'translocase' (Akita *et al.*, 1990; Geller *et al.*, 1993), we analyzed the Sec-dependency of the procoat protein mutants, all of which have a periplasmic loop of less than 25 residues. To our surprise, several of the mutants apparently required the Sec proteins for optimal translocation (Figure 9). This was very intriguing, because it was believed that it was solely the length of the segment to be translocated that was the determining

factor for Sec-dependent translocation (Kuhn, 1988; Andersson and von Heijne, 1993). For example, detailed studies of an 'inverted' leader peptidase revealed that the mechanism of insertion switched from Sec-independent to Sec-dependent when the length of the periplasmic domain was greater than 55 residues and that segments shorter than 25 residues are translocated absolutely independently of the Sec machinery (Andersson and von Heijne, 1993). Our studies reported here suggest that other features besides length can determine whether the Sec machinery is utilized. Taken together with the membrane potential results, we show that the potential-dependent transfer of negatively charged residues can occur both in a Sec-dependent and Sec-independent manner.

In conclusion, we feel there is a direct effect of the membrane potential on charged residues within the translocated region of membrane proteins. The interaction was found with leader peptidase constructs that have extended N-terminal tails and procoat protein mutants (−3 wild type and −4) which insert Sec-independently. For procoat proteins that depend on the SecA and SecY proteins (−5, −6, −3M and −4M) for optimal insertion, this may not be the case, because the potential effect may then be mediated by the Sec components.

Materials and methods

Strains and plasmids

Escherichia coli strains MC1061 [*ΔlacX174, araD139, Δ(ara, leu) 7697, galU, galK, hsr, hsm, strA*] and JM103 [*Δ(lacpro) thi, strA, supE, endA, sbcB, hsdR, traD, proAB, lacIqZM15*] were from our collection. CJ105 (*secAts51*) and CJ107 (*secYts24*) have been described (Wolfe *et al.*, 1985). The pQN-8 plasmid with the cloned M13 procoat protein gene, arabinose promoter and arabinose regulatory elements has been reported earlier (Kuhn and Wickner, 1985). Construction of the procoat−lep fusion (Kuhn *et al.*, 1986) and several derivatives thereof have been described (Kuhn *et al.*, 1990). Pf3−lep and its derivatives were expressed using the pING plasmid (Johnston *et al.*, 1985), which contains the arabinose promoter and arabinose regulatory elements.

Materials

Enzymes were purchased from Bethesda Research Laboratories. PMSF, CCCP and azide were from Sigma. Proteinase K was from Boehringer Mannheim. ^{35}S-translabel, a mixture of 85% [^{35}S]methionine and 15% [^{35}S]cysteine (1000 Ci/mmol) was from ICN K & K Laboratories. [^{35}S]dATP was from New England Nuclear.

DNA techniques

All enzyme reactions and DNA preparations were performed as described by Maniatis *et al.* (1982). Transfections and transformations followed the calcium chloride method of Cohen *et al.* (1973). Oligonucleotide-directed mutagenesis was performed by the procedure of Zoller and Smith (1983), with some modifications (Dalbey and Wickner, 1987). The DNA templates used to make the mutant constructs were M13 procoat−lep (Kuhn *et al.*, 1990) and Pf3−lep (Cao and Dalbey, 1994).

Protease mapping assay

One milliliter cultures of MC1061 bearing the pQN or the pING plasmids were grown to mid-log phase in M9 minimal media (Miller, 1972) with 0.5% fructose and 50 μg/ml of each amino acid but methionine. Arabinose (0.2% final concentration) was added to the M9 media to induce synthesis of the plasmid-encoded proteins. After 5 min, cells were labeled with 100 μCi ^{35}S-translabel for 1 min, chilled on ice and pelleted by centrifugation. After resuspending in 0.25 ml of ice-cold buffer A (0.1 M Tris−acetate, pH 8.2, 0.5 M sucrose, 5 mM EDTA), the cells were treated with lysozyme (80 μg/ml final concentration) and 0.25 ml of ice-cold water. Five minutes later, 150 μl of 0.2 M $MgSO_4$ was added to stabilize the spheroplasts and cells were again collected by centrifugation. The spheroplasts were resuspended in buffer B (50 mM Tris–acetate, 0.25 M sucrose, 10 mM $MgSO_4$) and incubated with or without proteinase K (1 mg/ml) for 1 h on ice. Another aliquot of spheroplasts was treated with 2% Triton X-100 and incubated with proteinase K. After quenching the protease with PMSF (5 mM final concentration), the samples were immunoprecipitated (Wolfe *et al.*, 1982) and analyzed by SDS−PAGE with a 15% polyacrylamide gel and then subjected to fluorography (Ito *et al.*, 1980). In Figures 6A and D, a 12% polyacrylamide gel was used with a different buffer (stacking gel, 0.5 M Tris−HCl, pH 6.8; separating gel, 1.5 M Tris−HCl, pH 8.8). For the azide and CCCP studies, the cells (1 ml cultures) were pretreated with drugs by the addition of 5 μl of CCCP (10 mM) for 45 s or by the addition of 30 μl of sodium azide (100 mM) for 1 min, prior to labeling of the cells.

Quantitation of the translocation data

Quantitation of the mature and precursor bands on the fluorograph was determined using a Technology Resource Inc. Line-Tamer PCLT300 scanning densitometer. The following equation was used for the determination of the percent processing of procoat−lep as two of the seven methionines were lost during conversion of the precursor protein to the mature product.

Processing (%) = (7/5 coat−lep x 100)/(7/5 coat−lep + procoat−lep)

Percent translocation of the leader peptidase derivatives was determined by quantitation of the appropriate bands from the fluorograph, as described above. Since only one methionine out of seven was lost during proteolysis of the translocated leader peptidase protein, the percent translocation was calculated as follows.

Translocation (%) = (7/6 digested products)/(7/6 digested products + non-digested protein)

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