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Efficient translocation of positively charged residues of M13 procoat protein across the membrane excludes electrophoresis as the primary force for membrane insertion

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The coat protein of bacteriophage M13 is inserted into the *Escherichia coli* plasma membrane as a precursor protein, termed procoat, with a typical leader peptide of 23 amino acid residues. Its membrane insertion requires the electrochemical potential but not the cellular components SecA and SecY. Since the electrochemical gradients result in the periplasmic side of the membrane being positively charged, the membrane potential could contribute to the transfer of the negatively charged central region of procoat across the membrane. Here we demonstrate that the central domain following the leader peptide can be translocated across the membrane even when the net charge of the region is changed from −3 to +3. This rules out an electrophoresis-like insertion mechanism for procoat. We also show that the *sec* independence of procoat insertion is linked to the presence of the second apolar domain. The deletion of most of the second apolar domain from a procoat fusion protein results in *sec* dependent membrane insertion of the hybrid protein. Moreover, like other proteins that require the *sec* genes, translocation of this *sec* dependent procoat protein is inhibited when positively charged residues are introduced after the leader peptide. Loop models involving one or two hydrophobic regions are presented that account for the differences in tolerance of positively charged residues.

Key words: membrane insertion/M13 coat protein/*sec* dependence

Introduction

While certain bacterial membrane proteins can insert directly into the membrane, most exported or membrane proteins use an insertion mechanism involving the *sec* gene products (Wickner, 1989). During, or shortly after their synthesis, some exported proteins first interact with cytoplasmic proteins, termed molecular chaperonins. This interaction maintains the exported protein in a loose conformation that is competent for membrane insertion (Randall and Hardy, 1986). The chaperonin exported protein complex is then thought to bind to the membrane associated SecA protein, where it is transferred to the SecY component under ATP consumption. The detailed mechanism by which the protein is actually translocated through the membrane still has to be elucidated at its molecular level.

The bacterial proteins that directly insert into the membrane include primarily small proteins such as the procoat protein of bacteriophage M13 (Wolfe *et al.*, 1985; Cobet *et al.*, 1989). The membrane insertion of the procoat protein has been extensively studied and it has been found that (i) procoat binds to the membrane via electrostatic interactions between the positively charged terminal regions of the protein and the acidic phospholipid head groups (Gallusser and Kuhn, 1990); (ii) procoat insertion occurs by the formation of a transmembrane loop (Kuhn, 1987) which requires the electrochemical membrane potential (Date *et al.*, 1980); (iii) procoat is cleaved to coat by leader peptidase after membrane insertion (Kuhn and Wickner, 1985). Despite numerous studies, the precise role of the electrochemical potential in transferring the acidic central region of procoat has remained elusive. One possible mechanism is an electrophoresis-like transfer of the negatively charged residues of the extracellular region of procoat, since due to the potential the outer face of the membrane is positively charged.

In the present study we investigated the role in protein insertion of the acidic residues of procoat at positions +2, +4 and +5. These residues were substituted with neutral or positively charged amino acids using site-directed mutagenesis. Surprisingly, when all three residues were replaced with positively charged arginines, the membrane insertion of procoat still occurred, although at a slower rate. This result is fundamentally different from those of other exported proteins, where a single positively charged residue inhibits membrane insertion (Li *et al.*, 1988; Yamane and Mizushima, 1988). The different effects of positive charges on the export of these proteins will be discussed in terms of the Sec machinery and loop models for protein translocation.

Results

The insertion of M13 procoat across the membrane does not require an acidic central region

To investigate whether the negatively charged residues in the translocated region of the M13 procoat protein have an essential function for membrane transfer, we generated a number of site-directed mutations in the region following the leader peptide. Figure 1A shows the mutations that were created and summarizes the translocation properties of the mutants, which were named according to the sequence of the first five amino acids of the mature region. Mutants were examined by pulse-labeling cells with [^{35}S]methionine, and analyzed by SDS−PAGE and fluorography. Wild-type procoat (Figure 1B, lane 1) and most of the mutants (lanes 2−7), which had been labeled during a 1 min pulse, were almost completely processed to coat protein during this time. A subtle retardation of processing was observed when a tryptophan was introduced at position +4 (lanes 4 and 5), but processing was even faster than wild-type when two asparagines replaced the aspartic acid residues at +4 and +5 (lane 6). Therefore, the reduction of the net charge from

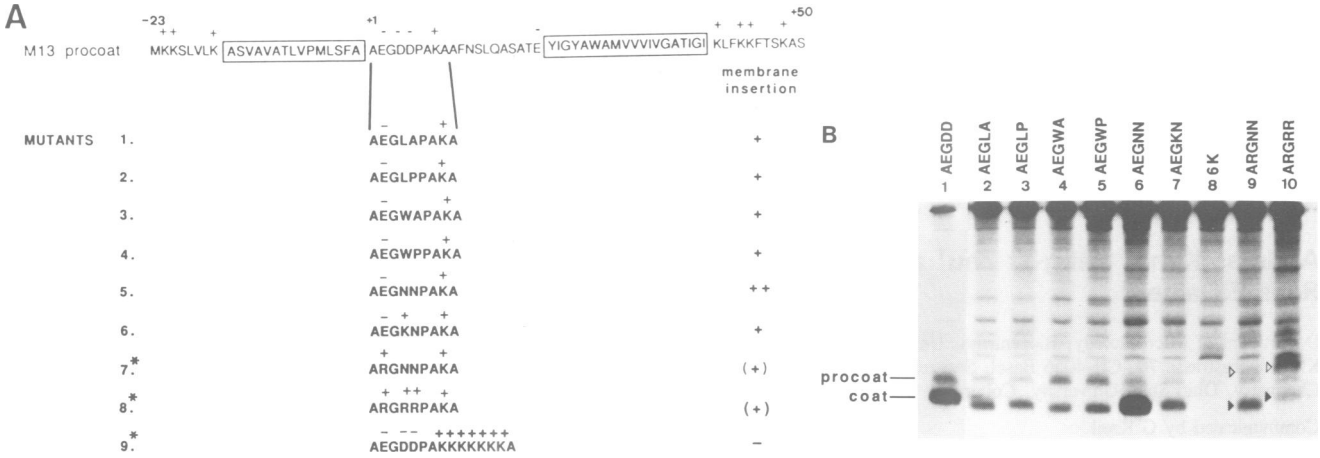

Fig. 1. (**A**) M13 procoat mutants and their membrane insertion properties. Substitutions (1–8) were made at positions +2, +4 and +5 in the central region of procoat. Procoat 6K has six lysines (9) inserted between residues +8 and +9. The asterisk marks the mutants that were studied with fusion proteins as well. These proteins were made by C-terminal extension of procoat with 103 amino acids derived from leader peptidase (lep). This modification of procoat does not interfere with its membrane insertion (Kuhn *et al.*, 1986a, 1987). (**B**) Processing of procoat mutants. *E.coli* HJM114 containing wild-type or mutant plasmids was grown to the mid-log phase in M9 medium containing 0.5% fructose supplemented with 19 amino acids (without methionine) at 37°C. After induction of the cells with IPTG (1 mM) for 15 min, the cells were incubated with [^{35}S]methionine for 1 min. The cells were then acid-precipitated and immunoprecipitated with antisera to coat protein. The samples were then analyzed by SDS–PAGE and fluorography. Procoat 6K (lane 8) is not recognized by the antibody. The arrowheads in lanes 9 and 10 correspond to procoat (▶) and coat protein (▷).

−2 to 0 in the region following the leader peptide has no inhibitory effect on membrane translocation of M13 procoat. Moreover, even increasing the net charge to +2 by arginyl substitution (lane 9), did not severely affect insertion kinetics.

Positively charged residues in the translocated region gradually slow down membrane translocation

Further increases in the net charge of the central region to +3 (lane 10) slowed the processing of procoat. Approximately 20% of procoat ARGRR was converted to the coat protein in a 1 min labeling. These results with procoat are in sharp contrast to those found with other proteins, where the introduction of a single arginine residue has a severe inhibitory effect on translocation (Li *et al.*, 1988; Yamane and Mizushima, 1988).

To quantify the extent of processing better and to avoid interference of the mutations with the antigenic recognition, we fused procoat at the C-terminal alanine to a 103 amino acid-long antigenic fragment of leader peptidase (lep). Previously, we have shown that the membrane insertion of the wild-type procoat fused to this fragment has normal kinetics and that the leader peptidase fragment remains on the cytoplasmic surface of the membrane (Kuhn *et al.*, 1986a). Wild-type and mutant procoat–lep fusions were pulse-labeled for 20 s and chased for either 5 s or 5 min (Figure 2). For the wild-type protein (procoat–lep AEGDD), the uncleaved form is not detectable at the earliest chase point (lane 1), indicating that it rapidly assembles across the membrane. The mutant procoat–lep ARGNN was processed at a slightly slower rate and showed ~20% unprocessed fusion protein at the 5 s chase (compare lanes 3 and 4). An even more pronounced retardation in processing was observed with the mutant ARGRR, showing 50% unprocessed form immediately following the pulse-labeling (lanes 5 and 6). However, the introduction of six additional lysinyl residues (procoat 6K–lep) completely inhibited the processing (lanes 7 and 8).

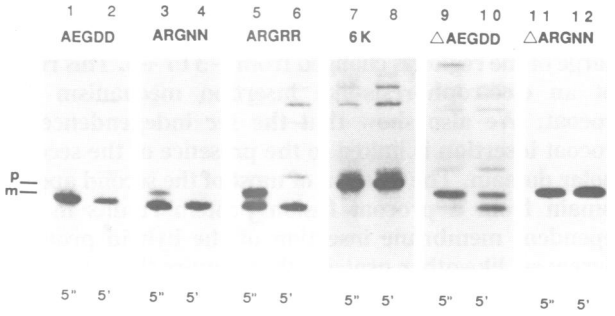

Fig. 2. Positively charged amino acids gradually inhibit membrane insertion. Procoat–lep fusion proteins with either the wild-type procoat sequence (lanes 1 and 2), ARGNN (lanes 3 and 4), ARGRR (lanes 5 and 6) or the 6K sequence (lanes 7 and 8) were expressed in *E.coli* LC137. The corresponding fusion proteins with either wild-type sequence (lanes 9 and 10) or ARGNN (lanes 11 and 12), both of which have procoat residues +27 to +48 deleted, were also studied. The p and m correspond to the precursor and mature form, respectively.

Positively charged residues are only translocated across the membrane when both hydrophobic regions of M13 procoat are present

To investigate whether the M13 procoat tolerates the four positively charged residues because its two hydrophobic regions favor the formation of a transmembrane loop, we deleted most of the second hydrophobic region (residues +27 to +48). As shown in Figure 2, this protein (Δprocoat–lep AEGDD) inserts across the membrane, though at a reduced rate (lanes 9 and 10). However, the introduction of one positively charged residue into this protein (Δprocoat–lep ARGNN) totally inhibited processing to the mature form (lanes 11 and 12).

The location of precursor and mature forms within the cell was analyzed by proteinase K accessibility (Figure 3). The cells were pulse-labeled with [^{35}S]methionine, the outer membrane was permeabilized with sucrose, Tris and EDTA

and digested with 1 mg/ml proteinase K for 60 min. The precursor forms of the mutants including procoat 6K−lep (lanes 4 and 5) and Δprocoat−lep ARGNN (lanes 10 and 11) were resistant to the proteinase, confirming that membrane translocation is the retarded step. For procoat−lep ARGRR (lanes 1−3) the mature form was clipped by the protease to a slightly shorter fragment, indicating that the C-terminal part encompassing the antigenic region was located in the cytoplasm. This had been observed for wild-type procoat−lep earlier (Kuhn *et al.*, 1986a). However, Δcoat−lep AEGDD (lanes 7−9) with only one hydrophobic region was entirely digested by the protease, confirming that the antigenic region had traversed the plasma membrane. The cellular conformations of the different mutants are summarized in Figure 4.

Positively charged residues inhibit membrane translocation of single-spanning, sec dependent proteins

To test whether the second hydrophobic region is essential for the *sec* independent translocation of procoat−lep, the plasmid encoding deletion mutant Δprocoat−lep AEGDD was transformed into the temperature sensitive strains CJ105 ($secA^{ts51}$) and CJ107 ($secY^{ts24}$). The transformants were grown at 30°C and then shifted to 42°C for 2 h. Portions were pulse-labeled with [^{35}S]methionine and analyzed by immunoprecipitation and SDS−PAGE (Figure 5). Procoat−lep ARGNN (lanes 1−3) was processed in all three strains to the mature form, whereas the OmpA protein accumulated in its precursor form in both CJ105 and CJ107. However, Δprocoat−lep AEGDD was converted to the mature form only in the HJM strain (lane 4), and not in the strains CJ105 (lane 5) and CJ107 (lane 6). We conclude that the membrane insertion pathways of procoat−lep and Δprocoat−lep differ fundamentally in respect to *sec* dependence.

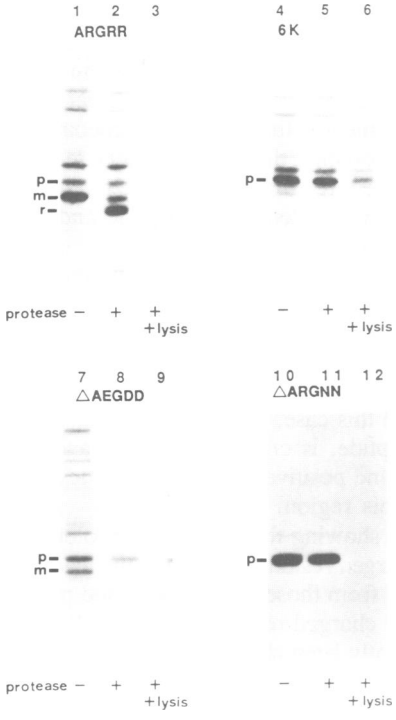

Fig. 3. Protease mapping of procoat mutants. *E.coli* HJM114 synthesizing procoat−lep ARGRR (lanes 1−3), procoat−lep 6K (lanes 4−6), △27−48 procoat−lep (lanes 7−9) or △27−48 procoat−lep ARGNN (lanes 10−12), was grown in M9 medium to the exponential phase and induced with 0.4% L-arabinose. The cells (0.5 ml) were pulse-labeled with [^{35}S]methionine (40 μCi) for 1 min, chased with methionine (500 μg/ml), and treated with 0.5 ml of buffer A (40% sucrose, 20 mM EDTA and 60 mM Tris, pH 8.0) to permeabilize the outer membrane. Samples were then incubated with or without proteinase K (1 mg/ml) for 60 min. Where indicated, a portion of cells was treated with 2% Triton X-100 to lyse the cells prior to proteinase treatment. The samples were analyzed by acid-precipitation, immunoprecipitated with antisera to leader peptidase, and subjected to SDS−PAGE and fluorography. The p, m and r correspond to the precursor, mature form, and the protease resistant fragment, respectively.

Discussion

Electrophoretic models of protein export propose that charged domains of proteins are moved across the membrane by the electric field. The M13 procoat protein provides very useful features to test this proposal directly. First, it requires the membrane potential (Date *et al.*, 1980). In *Escherichia coli*, the membrane potential is such that there is a net positive charge on its outer surface and a net negative charge on the cytoplasmic side. Second, the region of the procoat protein which is translocated across has a net charge of −3. Third, the M13 procoat protein inserts into the membrane

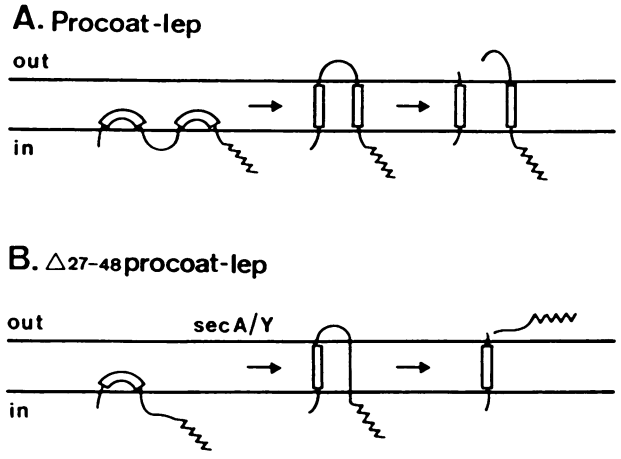

Fig. 4. Loop models of protein translocation depicting (**A**) the *sec* independent and (**B**) the *sec* dependent modes of procoat insertion. The lep region corresponds to the zig-zag line.

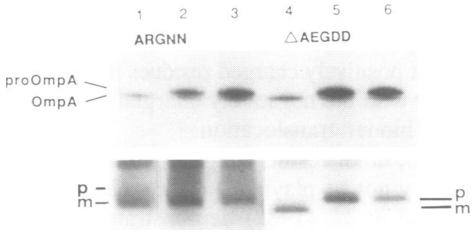

Fig. 5. Membrane insertion of △procoat−lep requires *secA* and *secY*. *E.coli* HJM 114 (lanes 1 and 4), CJ105 (lanes 2 and 5), and CJ107 (lanes 3 and 6) synthesizing procoat−lep ARGNN (lanes 1−3) or △27−48 procoat−lep AEGDD (lanes 4−6) were grown at 30°C to a density of 5×10^7 cells/ml. After a temperature shift to 42°C for 2 h, 0.4% arabinose was added for 30 min. The cells were labeled with 10 μCi [^{35}S]methionine for 5 min. The samples were acid-precipitated and immunoprecipitated with antisera to OmpA (upper panel) and to leader peptidase (lower panel), as described in Figure 1B. The p and m correspond to the precursor and mature forms, respectively.

as a loop structure (Kuhn, 1987) involving both hydrophobic regions which flank the extracellular domain (Kuhn *et al.*, 1986b). This negatively charged domain is therefore transported to the positively charged periplasmic membrane surface.

Our results reported here exclude an electrophoresis-like mechanism for the translocation of the M13 procoat protein. The procoat protein can transfer its central region across the membrane even when it has positively charged residues in the translocated region. Thus, the net charge of the procoat's central region and the electrical field do not move the protein across the membrane. Moreover, it has been shown previously that the chemical potential can substitute for the electrical component of the membrane potential (Bakker and Randall, 1984). Therefore, the requirement for an electrochemical potential during protein insertion has to have another purpose. In addition, we do not know for procoat, of course, whether the acidic and basic residues are translocated across the membrane in a truly charged or in a neutralized form surrounded by a water shell. Our results with the various procoat mutants that contain less ionic residues than the wild-type gave no clear conclusion, since some translocated faster but others slower than the wild-type protein.

Although the membrane potential is not directly involved in the insertion process, it might have an important role for the orientation of the inserting protein. It has been suggested earlier that proteins orient themselves according to the charged groups flanking the hydrophobic leader peptide region (Daniels *et al.*, 1981; von Heijne, 1986a,b). The procoat molecule generates a favorable charge distribution even in the case of the mutant procoat (with four positively charged residues in the central region), since the cytoplasmic portions of the protein contribute seven positively charged residues.

While M13 procoat tolerates positively charged residues in the central domain, we observe a kinetic effect on membrane translocation (Figure 2). One possible reason for this is that the electric field has a minor effect on the translocation kinetics and positively charged residues slow the translocation. Another possibility is that the positively charged residues in this region interact electrostatically with the negatively charged membrane surface and hinder the translocation of this region. We favor this latter possibility since neutral residues promote faster insertion. Previously, we have shown that the M13 procoat electrostatically binds to the membrane surface by its positively charged N and C-terminal regions (Gallusser and Kuhn, 1990). Thus, it is plausible that positively charged residues in the central region also interact with the negatively charged phospholipid head groups and hinder translocation.

In addition, other results suggest that the electrochemical potential may not be playing a direct role in translocation. Zimmermann *et al.* (1982) demonstrated that membrane insertion was dramatically less dependent on the membrane potential with a procoat mutant with a mutation at +2. It is difficult to explain this result by an electrophoretic transfer mechanism. Possibly, the membrane potential acts indirectly, like on the packing of the phospholipids or on the precursor conformation. Furthermore, *in vitro* studies showed that the requirement of a membrane potential differed among various precursor proteins (Yamada *et al.*, 1989a) and assay conditions (Yamada *et al.*, 1989b). For example, the requirements for a membrane potential was modulated by the amount of the SecA protein added. This suggests that the membrane potential is rather involved in the binding of the precursor to the inner surface of the membrane. M13 procoat requires for its initial binding a negatively charged inner membrane surface to which the membrane potential contributes. It is therefore plausible that a dissipation of the potential also hinders the procoat protein from binding to the membranes.

In the present study, we also tested whether the *sec* independent assembly of procoat was due to the second apolar domain of procoat, which participates in forming the loop structure which then inserts the central region across the lipid bilayer. Previously, we showed that procoat fused with a 103 amino acid segment derived from the polar, C-terminal domain of leader peptidase inserts into the membrane in a *secY* independent manner (Kuhn *et al.*, 1986b). Our results here indicate that when most of the second apolar domain of procoat is deleted, then the fusion protein assembles at a reduced rate in a *secY* dependent manner. This establishes that the independence of *secY* is due to the presence of the second apolar region of procoat. However, when the two apolar regions are far apart, separated by a long stretch of ~ 100 amino acid residues, they lose their capacity to promote *sec* independent insertion. For example, a procoat fusion protein with 98 residues derived from the OmpA sequence inserted only in the presence of functional SecA and SecY (Kuhn, 1988). Taking these results together, we suggest that the determining factor for *sec* requirement is the size of the procoat domain to be translocated. Possibly, the Sec machinery recognizes in the preprotein some three-dimensional structure comprising the already laterally inserted leader peptide and the adjoining polar stretch within the mature domain (Figure 4B).

The *sec* independent protein insertion might therefore be based on a synergistic entry of both hydrophobic regions of the procoat protein into the lipid bilayer (Figure 4A). When parts of the mature hydrophobic region are deleted, the formation of a transmembrane loop has to proceed differently. In this case, the protein sequence following to the leader peptide, is critical for membrane translocation (Figure 4B), and positively charged residues are no longer tolerated in this region.

Our results showing that procoat can tolerate up to four positively charged residues following the leader peptide are very different from those of other secreted proteins, where one positively charged residue blocks membrane insertion. This has recently been shown for alkaline phosphatase (Li *et al.*, 1988), lipoprotein-β-lactamase (Yamane and Mizushima, 1988) and OmpA (Zhu,H., Kuhn,A. and Dalbey,R., in preparation). It is interesting to note that exported proteins very rarely have a positive charge in the C-terminal part of the leader peptide or in the first few positions of the mature sequences (von Heijne, 1986a). Why do positively charged residues have such an inhibitory effect on all other exported proteins but not on M13 procoat? One possibility is that these positive charges prevent the proteins from interacting with the secretory apparatus (e.g. with the SecA protein). Therefore, a *sec* dependent procoat would be very sensitive to positively charged residues, as shown here for the *sec* dependent procoat mutant ΔARGNN. Thus, it seems that Sec interactions require certain structural features of the mature sequence.

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