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Initial steps in protein membrane insertion. Bacteriophage M13 procoat protein binds to the membrane surface by electrostatic interaction

Andreas Gallusser and Andreas Kuhn

Microbiology Department, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

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Bacteriophage M13 procoat protein is synthesized on free polysomes prior to its assembly into the inner membrane of *Escherichia coli*. As an initial step of the membrane insertion pathway, the precursor protein interacts with the cytoplasmic face of the inner membrane. We have used oligonucleotide-directed mutagenesis to study the regions of the procoat protein involved in membrane binding. We find that there is an absolute requirement for positively charged amino acids at both ends of the protein. Replacing these with negatively charged residues resulted in an accumulation of the precursor in the cytoplasm. We propose that the positively charged amino acids are directly involved in membrane binding, possibly directly to the negatively charged phospholipid head groups. This was tested *in vitro* with artificial liposomes. Whereas wild-type procoat interacted with these liposomes, we found that procoat mutants with negatively charged amino acids at both ends did not bind. Therefore, we conclude that newly synthesized M13 procoat protein binds electrostatically to the negatively charged inner membrane of *E.coli*.

Key words: liposomes/M13 procoat/protein membrane interaction/signal peptide

Introduction

Targeting of proteins to cellular membranes might occur by several distinct mechanisms (Wickner and Lodish, 1985). While some proteins transported to the nucleus use a signal-receptor mechanism (Richardson *et al.*, 1986), transport to the endoplasmic reticulum seems to occur by a series of steps which each show a broad substrate specificity (Walter, 1987). In general, nascent secretory and plasma membrane proteins first associate with the signal recognition particle (SRP) directing the protein to the endoplasmic reticulum membrane. Following the interaction of the ribosome with the membrane associated docking protein (DP), the precursor protein is released from the complex and inserted into the membrane possibly via interaction with the integral signal sequence receptor (SSR) (Wiedmann *et al.*, 1987). Besides this general pathway, the various proteins and cellular systems show a broad range of requirements including obligatory coupling to ongoing translation or dispensability of the signal recognition particle (Zimmermann and Meyer, 1986). Small proteins, like prepromellitin or prepropeptide Gla do not require SRP and DP and presumably insert into the membrane by a simple mechanism (Zimmermann and Mollay, 1986; Schlenstedt and Zimmermann, 1987).

In bacteria a similar cascade involving numerous cellular components necessary for membrane protein insertion has been found (Oliver and Beckwith, 1982; Akiyama and Ito, 1985; Collier *et al.*, 1988; Crooke *et al.*, 1988; Lill *et al.*, 1988). However, some proteins, like M13 procoat, also insert into the membrane in the absence of these components (Wolfe *et al.*, 1985). We have recently shown using gene fusion techniques that the extracellular region of M13 procoat protein determines the lack of requirement for the components SecA and SecY of the bacterial secretion system (Kuhn, 1988). Simple protein segments might translocate across the membrane in a spontaneous fashion.

We are interested in understanding how these proteins can bypass the complex insertion machinery and efficiently translocate across the membrane. The extracellular segment of M13 procoat is flanked by two hydrophobic regions which both contribute to the insertion of the protein into the membrane (Kuhn *et al.*, 1986b). Although hydrophobic partitioning of M13 procoat might explain its spontaneous membrane insertion, other hydrophilic regions determine its targeting to the membrane. We have previously found that membrane insertion of procoat is blocked when the C-terminal region of procoat is altered (Kuhn *et al.*, 1986a). The hydrophilic and basic character of this region suggests that it might be involved in the binding of the newly synthesized protein onto the membrane surface. We show here that both the N- and C-terminal basic regions of the M13 procoat protein contain membrane targeting information and allow the protein to interact electrostatically with the cytoplasmic membrane surface.

Results

Aminoterminal residues −22 to −16 of M13 procoat protein are required for membrane insertion

To investigate the role of the positively charged residues at the N-terminal end of M13 procoat protein, we constructed a set of deletion mutants (shown in Figure 1) by oligonucleotide-directed site-specific mutagenesis. Amino acid residues −22 to −16 including three lysyl residues were deleted which changed the charge of the N-terminal region from +3 to 0. In addition, the alanine residue at position −15 was substituted by an asparagine residue. This replacement was designed to introduce a unique *Eco*RI restriction site (Figure 1A) which was used for further mutations at the N-terminal end of procoat.

A similar deletion mutant, ΔC30, at the C-terminal end of procoat was described earlier (Kuhn *et al.*, 1986a). The two mutants ΔN30 and ΔC30 were combined in a double deletion mutant ΔN30/C30 (Figure 1A). The net charge of the procoat protein was thus changed from +4 to −3 and, in addition, the overall hydrophobicity increased. The plasmids encoding wild-type procoat, ΔN30, ΔC30 and ΔN30/C30 were transformed into *Escherichia coli* LC137 and the synthesis of wild-type procoat or the various deletion

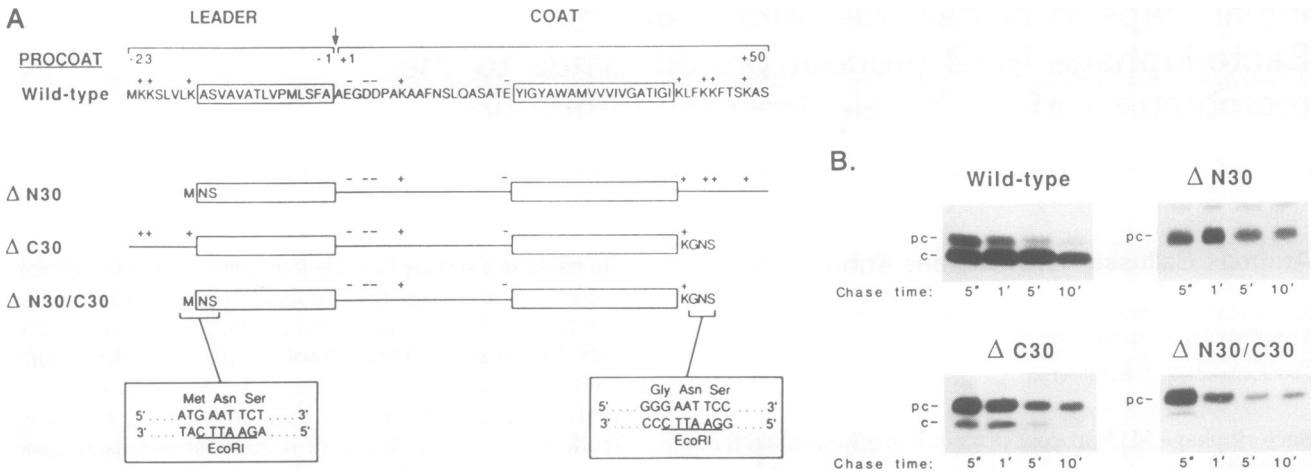

Fig. 1. Procoat mutants with terminal deletions are inhibited for processing *in vivo*. (**A**) The amino terminal deletion of ΔN30 removes amino acid residues −22 to −16 and replaces the alanine residue at position −15 by an asparagine residue. For mutant ΔC30 the C-terminal 10 residues were replaced by the sequence Gly-Asn-Ser. The precursor of the double mutant ΔN30/C30 contains 59 amino acids with both terminal regions deleted. The *Eco*RI restriction sites created with the deletions at both terminal regions are shown in rectangles. (**B**) Cultures of *E.coli* LC137 with plasmids encoding wild-type procoat or the various deletion mutants were grown overnight at 30°C in M9 minimal medium containing ampicillin and 0.5% fructose. Fresh overnight cultures were diluted 1:50 into M9 minimal medium and grown at 30°C to a density of 2×10^8 cells/ml. The cultures were induced by the addition of 0.5% L-arabinose for 15 min, then pulse-labelled for 1 min with 10 μCi of [^{35}S]methionine and chased with 1 mg/ml non-radioactive L-methionine for the indicated times. The samples were precipitated with 20% TCA, immunoprecipitated with antibodies to M13 coat protein and analysed by SDS−PAGE and fluorography (Ito *et al.*, 1980).

mutants was induced. The newly synthesized proteins were pulse-labelled with [^{35}S]methionine for 1 min and then chased with an excess of non-radioactive methionine. Samples were analysed for procoat and coat protein by immunoprecipitation, SDS−PAGE and fluorography (Figure 1B). Since cleavage of the leader sequence from the precursor by the leader peptidase occurs at the periplasmic face of the inner membrane (Wolfe *et al.*, 1983), processing from procoat to coat can be considered as an indication of membrane insertion.

While all three deletion mutants were cleaved *in vitro* with purified leader peptidase (data not shown) processing *in vivo* was strongly inhibited. Small amounts of the processed coat protein could only be detected with ΔC30. This is possibly due to the function of the one remaining lysyl residue at position 40.

Although the mutant proteins were synthesized in a strain lacking the cytoplasmic *lon* protease, the deletion mutants were degraded. Particularly, the double deletion ΔN30/C30 was rapidly hydrolysed. This susceptibility is clearly different to procoat mutant proteins that are inserted across the cytoplasmic membrane (Kuhn and Wickner, 1985).

Substitution of positively charged amino acids restores membrane insertion

Since short deletions at the N- or C-terminal region resulted in a loss of membrane insertion capability, various peptides were introduced to study restoration of the function. Pairs of complementary oligonucleotides were hybridized and ligated into the *Eco*RI restriction sites of either ΔN30 or ΔC30. The amino acid sequences of oligonucleotide-directed insertion mutants are summarized in Figure 2. The basic character of the two terminal regions was restored by arginyl and lysyl residues. Membrane insertion and processing of these mutants was studied by pulse−chase experiments (shown in Figure 2).

In the N-terminal mutant ΔN38 a peptide of six amino acid residues including three arginyl residues was introduced between the methionine and asparagine residues. The mutant protein was processed to coat with slightly slower kinetics than the wild-type procoat (Figure 1B). To see if an increase in positive charge at the N-terminus would increase the efficiency of membrane insertion, two oligonucleotides were introduced in tandem resulting in a protein with 12 additional amino acids including six arginyl residues. The mutant ΔN38/38 was processed to coat with an efficiency quite comparable with ΔN38.

It is possible that the arrangement of the positively charged residues is crucial for maximum activity of membrane insertion. This was investigated with the C-terminal region of procoat. By NMR spectroscopy it has been shown that the region +40 to +50 is α-helical (Cross and Opella, 1985). Assuming an α-helical structure, the three positively charged residues were either distributed (ΔC36 and ΔC38) or arranged on one side (ΔC37) forming an amphiphilic helix. The amino acid sequences of the C-terminal region of these mutants are shown in Figure 2B.

Cells synthesizing the procoat mutants ΔC36, ΔC37 and ΔC38 were pulse-labelled with [^{35}S]methionine for 1 min and chased with an excess of non-radioactive methionine (Figure 2B). At different chase times, samples were analysed as described above. Surprisingly, all three mutants shared the same features of membrane insertion and processing within a chase time of 10 min.

During synthesis the mutants were inserted into the cytoplasmic membrane and processed quite efficiently, comparable with wild-type procoat. However, for longer chase times a portion of the procoat proteins was no longer competent for proper membrane insertion and remained unprocessed. The processed coat proteins of the three mutants, on the other hand, were rapidly degraded with a half-life of < 1 min. Thus, the mature mutant proteins are much more susceptible to proteolysis than the wild-type coat protein ($t_{1/2} = 4$ min).

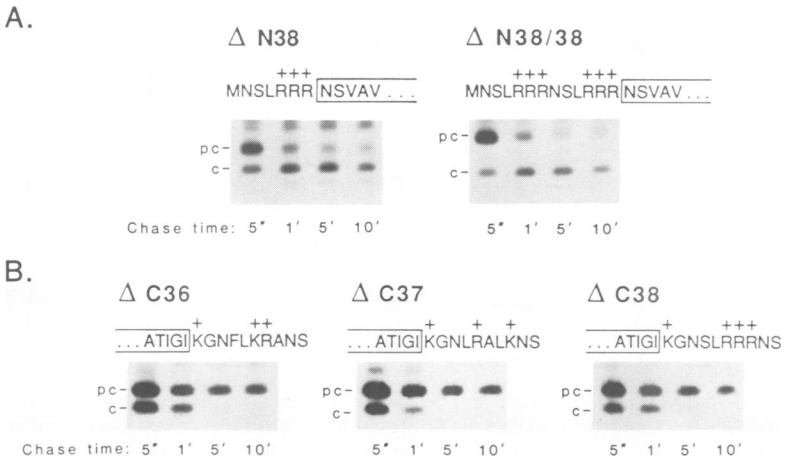

Fig. 2. Altered terminal regions partially restore membrane insertion. Short DNA fragments coding for positively charged residues were ligated into the *Eco*RI restriction sites of the deletion mutants ΔN30 and ΔC30. Plasmids coding for the N-terminal procoat mutants ΔN38 and ΔN38/38 (**A**) and for the C-terminal procoat mutants ΔC36, ΔC37 and ΔC38 (**B**) were transformed into *E.coli* LC137 and *in vivo* processing was analysed in pulse–chase experiments as described in Figure 1B. The amino acid sequence of the altered region is shown on top of the corresponding pulse–chase experiment.

Procoat mutants which change the charge but keep the polarity in the N- and C-terminal regions are not translocated across the membrane

The removal of basic amino acid residues at the amino terminus of a leader sequence decreased the efficiency of membrane insertion, as shown earlier for a number of other membrane proteins (Inouye *et al.*, 1982; Vlasuk *et al.*, 1983). For M13 procoat protein, both positively charged terminal regions might be directly involved in an electrostatic interaction of the precursor with the negatively charged surface of the inner membrane. To test this we constructed further mutants with altered N- and C-terminal regions. The basic amino acid residues were replaced by negatively charged residues. Two complementary oligonucleotides which code for three neutral and three acidic amino acid residues were introduced into the *Eco*RI restriction sites of ΔN30 or ΔC30, respectively. The amino acid sequences of the resulting procoat mutants ΔN39 and ΔC39 are shown in Figure 3A. Using recombinant DNA techniques we isolated a double mutant ΔN39/C39 with two negatively charged terminal regions.

The effect of the reverse charge at both terminal regions on membrane insertion was assayed by pulse–chase experiments (Figure 3B). None of the mutant proteins, ΔN39, ΔC39 and ΔN39/C39, were processed to the mature form within 10 min. The block in processing might be due either to lack of membrane insertion or to inhibition of processing by leader peptidase. *In vitro* processing with leader peptidase showed that all three mutants were normal substrates for this enzyme (data not shown). To investigate the location of the mutant proteins, cells synthesizing the mutant proteins were pulse-labelled, then rapidly chilled and treated with sucrose, Tris and EDTA to permeabilize the outer membrane and proteinase K was added for various times. As shown in Figure 4, the procoat mutant proteins were inaccessible to the protease, whereas the outer membrane protein A (OmpA) of the same cells was digested (Figure 4, lanes 1–4), confirming that the outer membrane had been permeabilized. Under the same conditions the cytoplasmic protein araB which serves as a control was also stable (not shown). A portion of the cells was lysed with Triton X-100 (lane 5) and the procoat as well as the control proteins were completely digested. Similarly, the procoat protein accumulated in the deletion mutants ΔN30, ΔC30 and ΔN30/C30 was not accessible to externally added protease (data not shown). The results shown in Figures 3 and 4 clearly indicate that negatively charged amino acid residues, at one or both terminal regions of procoat protein, inhibit the translocation of the protein across the membrane. Possibly, these acidic amino acid residues prevent the interaction of the precursor with the inner membrane and thereby block membrane insertion.

We therefore analysed whether the mutant precursor proteins were associated with the inner membrane. Cells with plasmids coding for wild-type procoat and the mutants ΔN39, ΔC39 and ΔN39/C39 were pulse-labelled for 1 min with [^{35}S]methionine. The membranes were extracted with 100 mM NaOH followed by a centrifugation. Aliquots of the resulting supernatant and pellet fractions were immunoprecipitated with antisera to coat protein, to ribulokinase or lipoprotein as controls for a cytoplasmic and a membrane protein, respectively. Wild-type procoat was found in the pellet fraction (Figure 5A, lane 3) whereas the mutant precursors ΔN39, ΔC39 and ΔN39/C39 with negatively charged terminal regions were preferentially localized in the soluble fraction (Figure 5B, lanes 2, 5 and 8). Similar results were obtained with the deletion mutants ΔN30, ΔC30 and ΔN30/C30 (Figure 5C). We conclude that these mutants are weakly membrane associated, whereas procoat H9, a mutant of the proline residue at position −6 in the leader peptidase target site (Kuhn and Wickner, 1985), was only found in the pellet fraction (Figure 5A, lane 6). The H9 procoat protein accumulates in a transmembrane configuration. We applied the same fractionation procedure to procoat mutants with altered hydrophobic regions. As previously shown these mutants do not assume a transmembrane configuration (Kuhn *et al.*, 1986b). Mutant OL8 with an arginyl residue at position −8 in the hydrophobic region of the leader, mutant OM30 with an arginyl residue at position +30 of the coat membrane anchor domain and the corresponding double mutant OL8/OM30 were tested (Figure 5D). We found that the procoat mutants OL8 and OM30 were tightly associated with the membrane whereas OL8/OM30 showed a weak

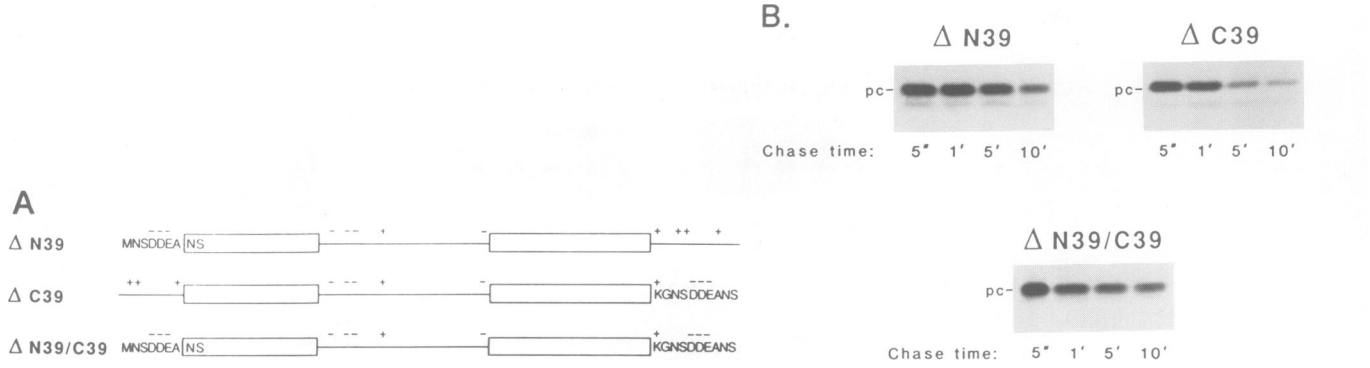

Fig. 3. Negatively charged amino acid residues at both terminal regions inhibit *in vivo* processing of procoat. (**A**) Two prehybridized oligonucleotides coding for the sequence Ser-Asp-Asp-Glu-Ala were introduced into the *Eco*RI restriction site of the deleted procoat mutants ΔN30 and ΔC30, respectively. The resulting procoat mutants ΔN39 and ΔC39 were combined to the double mutant ΔN39/C39 with negatively charged amino acid residues at both terminal regions. (**B**) Exponentially growing cultures of *E.coli* LC137 bearing the mutant plasmids were pulse-labelled with [^{35}S]-methionine for 1 min and chased with 1 mg/ml of non-radioactive L-methionine for the indicated times. Samples were analysed by immunoprecipitation, SDS−PAGE and fluorography.

membrane association similar to that observed for ΔC39. Although these mutant proteins cannot translocate across the membrane, a partial partitioning of the hydrophobic domains into the bilayer obviously still occurs. Thus, the initial step of membrane binding supported by the positively charged terminal regions was not affected by the mutations.

We devised a test to show directly an interaction of the terminal regions of procoat with the membrane surface. The double mutant ΔN39/C39 and the wild-type protein were assayed in an *in vitro* system for their binding to artificial liposomes. The procoat genes were put under a bacteriophage SP6 promoter and transcribed *in vitro* with purified SP6 polymerase. *In vitro* translation of the transcripts with a reticulocyte lysate was carried out in the presence of liposomes with a negatively charged surface. These liposomes were prepared using a mixture of 75% of the zwitterionic phosphatidylcholine (PC) and 25% of the negatively charged phosphatidylglycerol (PG). 25% PG content corresponds to the composition of the *E.coli* inner membrane (Burnell *et al.*, 1980). After *in vitro* translation the reaction mixture was adjusted to 50% sucrose and the liposomes were separated from the translation reaction mixture by floating through a sucrose step gradient (Figure 6). An equilibrium with >90% of the liposomes in the top fraction was achieved after 40 min centrifugation. The fractions of the gradient were analysed by SDS−PAGE and fluorography (Figure 7). In the absence of liposomes, wild-type procoat protein and the mutant ΔN39/C39 were found in the bottom fractions (Figure 7, lane 1). However, in the presence of PG-containing liposomes, wild-type procoat comigrated with the phospholipids and was detected in the top fractions (Figure 7A, lane 4) indicating binding of the protein to the liposomes. In contrast to the wild-type precursor, the mutant ΔN39/C39 did not associate with the negatively charged surface of the liposomes and remained in the bottom fraction (Figure 7B, lane 3).

Binding of the mutant protein ΔN39/C39 was restored when the membrane surface was no longer negatively charged by using only the zwitterionic phosphatidylcholine (PC) to generate the liposomes (Figure 7, lanes 5 and 6). These results confirmed our presumption that binding of procoat protein to negatively charged phospholipid membranes is promoted by the positively charged amino acid residues at both terminal regions.

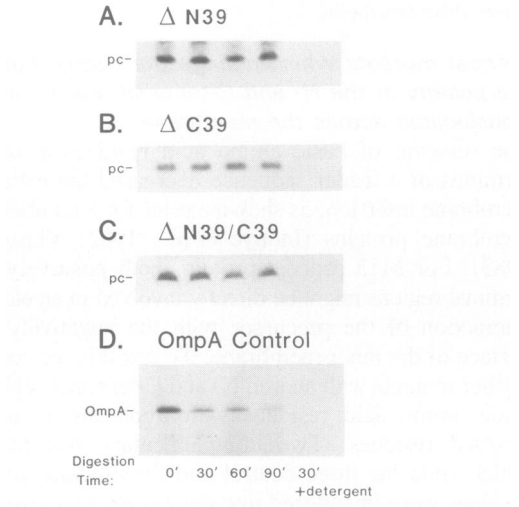

Fig. 4. Procoat mutants with negatively charged terminal regions are not inserted across the membrane. 0.5 ml of an exponentially growing culture of *E.coli* LC137 with plasmids encoding procoat mutants ΔN39, ΔC39 or ΔN39/C39 were induced with 0.5% L-arabinose for 15 min and labelled with 50 μCi [^{35}S]methionine for 1 min. The cells were osmotically shocked by mixing with 2 vol of ice cold 60 mM Tris−HCl pH 8, 40% sucrose and 20 mM EDTA, and incubated on ice for 10 min. Proteinase K was added to a final concentration of 1 mg/ml and the samples were incubated on ice for the indicated times. A portion of the cells was lysed by adding 2% Triton X-100 prior to proteolysis. After digestion, the proteinase was inactivated by adding phenylmethanesulfonylfluoride and the samples were precipitated by 20% TCA. All samples were immunoprecipitated with antibodies to coat (A, B and C) and to the outer membrane protein OmpA (D) and assayed by SDS−PAGE and fluorography.

Discussion

The results presented in this paper show that M13 procoat protein requires the positively charged amino acid residues at the amino and carboxy termini to initiate its insertion into the membrane. Procoat mutants lacking the positively charged amino acids or containing negatively charged residues in their place accumulated in the cytoplasm of the cell. This was concluded from three lines of evidence: the accumulated proteins (i) were not accessible to externally added protease, (ii) were not processed by leader peptidase to their mature forms although they were substrates for this

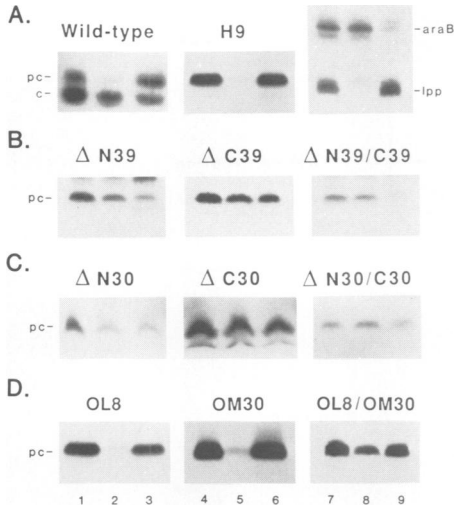

Fig. 5. Altered terminal regions of procoat affect its cellular location as analysed by cell fractionation. Total cell fractions (lanes 1, 4 and 7), supernatant fractions (lanes 2, 5 and 8) and pellet fractions (lanes 3, 6 and 9) were analysed for (**A**) wild-type procoat (lanes 1–3) and the cleavage mutant H9 (lanes 4–6), (**B**) mutants with altered terminal regions ΔN39, ΔC39 and ΔN38/C39, (**C**) deletion mutants ΔN30, ΔC30 and ΔN30/C30, and (**D**) mutants with arginine substitutions in the hydrophobic regions OL8, OM30 and OL8/OM30. Cultures of *E.coli* LC137 bearing the various plasmids were induced with 0.5% L-arabinose for 15 min. Following a 1 min pulse with [^{35}S]methionine, the cultures were lysed by adding an equal volume of ice cold 0.2 N NaOH. Half of each sample was precipitated with 20% TCA for the total cell protein (lanes 1, 4 and 7). The second half of the sample was centrifuged and the supernatant (lanes 2, 5 and 8) and the pellet (lanes 3, 6 and 9) were precipitated with TCA. All fractions were analysed for procoat and coat by immunoprecipitation, SDS–PAGE and fluorography. As a control, all the samples were analysed for the location of the cytoplasmic ribulokinase (araB) and the outer membrane lipoprotein (lpp) A, lanes 7–9).

enzyme *in vitro* and (iii) did not fractionate with the cell membrane under alkaline conditions.

Other export-defective procoat mutants (Kuhn *et al.*, 1986b) which contained the terminal positively charged residues but had altered hydrophobic regions (OL8 and OM30) were, however, found in the membrane fraction. Similar results were obtained with a mutant of the maltose binding protein (MBP) which has an altered hydrophobic region in the leader. Although this mutant was deficient for membrane translocation, it was associated with the membrane (Thom and Randall, 1988). We found that the procoat mutants OL8 and OM30 remain associated with the membrane after an extraction procedure at alkaline pH. This suggests that these mutant proteins are partitioned into the membrane and held by hydrophobic interactions even though they are inhibited from assuming a transmembrane configuration.

In general, membrane binding of proteins is difficult to assess *in vivo*. Fractionation experiments show that cytoplasmic proteins may artificially bind (Koshland and Botstein, 1982) or integral membrane proteins may dissociate from the membrane during the isolation procedure (Russell and Model, 1982). Indeed, we observed that a major portion of the mature coat protein was in the soluble fraction under alkaline conditions, although we know by other techniques that its only location is the inner membrane. Hence, this method can only be applied to tightly associated proteins. Wild-type procoat, however, was mainly found in the membrane pellet. Even after a short pulse of radiolabelled methionine, most of the procoat appeared with the membrane. This suggests that after synthesis, procoat rapidly binds to the surface of the cell membrane.

Membrane binding of double mutant ΔN39/C39 was tested *in vitro* with artificial liposomes. Wild-type procoat was bound to these liposomes which were added during synthesis. However, the procoat mutant with both terminal regions negatively charged did not bind to the negatively charged liposomes. When the net charge of the lipid components was changed to neutral, binding of procoat ΔN39/C39 to the liposomes was restored, suggesting that the precursor is no longer electrostatically repelled from the lipid surface. A direct involvement of the membrane lipids in membrane insertion has been observed previously using an *E.coli* strain with an altered lipid composition accumulating non-processed PhoE (de Vrije *et al.*, 1988). The results obtained *in vivo* are therefore consistent with these *in vitro* results which suggests that the procoat protein interacts directly with the lipid components of the membrane and not with a protein receptor. Indeed, procoat protein inserts into liposomes without any other protein (Ohno-Iwashita and Wickner, 1983; Geller and Wickner, 1985). In contrast to other precursor proteins, membrane insertion of procoat does not require SecY function, one protein component of the proposed membrane insertion machinery (Akiyama and Ito, 1985). Procoat might simply bind by electrostatic interaction to the negatively charged phospholipid head groups. However, many other cytoplasmic proteins have positively charged residues exposed and might therefore electrostatically interact with the membrane surface. Presumably, these interactions occur, but they are short-lived and weak. We propose that for a tight membrane association, additional features of a protein are required, such as hydrophobic regions which are able to partition into or span the lipid bilayer. Our results shown here indicate that there is no specific sequence of charged amino acids required for membrane binding. The charged residues might be distributed or facing one side of an α-helix, and lysyl or arginyl residues were indistinguishably accepted.

The targeting function undertaken by both terminal regions of procoat might substitute for targeting mechanisms present in other bacterial membrane proteins (Lill *et al.*, 1988; Cunningham *et al.*, 1989). We propose that simple proteins, like M13 procoat, are targeted to the membrane by electrostatic interaction, while more complex proteins require additional proteinaceous components. However, other bacterial precursor proteins also contain positively charged residues in the amino-terminal region (n-region) in the leader sequence (von Heijne, 1986). For a few of them, these positively charged residues had been altered. Substitution of the positively charged residues by negatively charged amino acids lowered the rate of processing for lipoprotein (Inouye *et al.*, 1982; Vlasuk *et al.*, 1983), staphylokinase (Iino *et al.*, 1987) or for the outer membrane protein PhoE (Bosch *et al.*, 1989). In addition, deletion of nearly the entire leader sequence of maltose binding protein inhibited its export (Bedouelle *et al.*, 1980) and it was recently shown that it does not bind to the membrane (Thom and Randall, 1988). Taken together, these results show that the positive charges in the amino-terminal region of the leader sequence are required to initiate membrane insertion of the protein.

The various regions of the leader peptide (von Heijne,

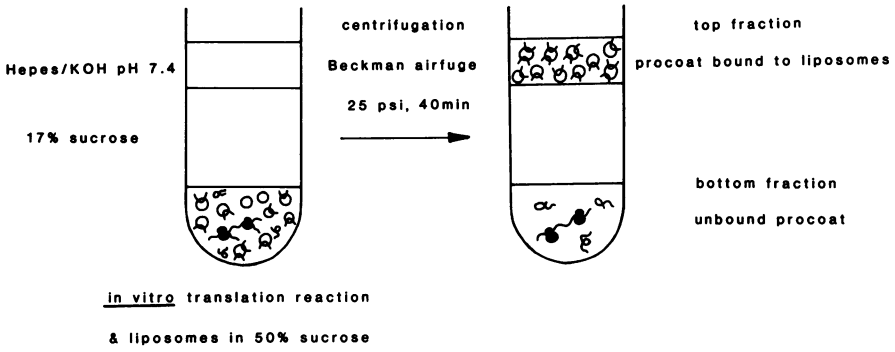

Fig. 6. Binding assay of procoat protein to liposomes.

1986) clearly have distinct functions. The positively charged n-regions are involved in initiating membrane insertion by binding to the membrane surface. The hydrophobic h-region is required for partitioning into the core of the bilayer and to translocate across the bilayer by formation of a transmembrane loop (Kuhn, 1987). Finally, the hydrophilic c-region serves as a recognition site for the processing enzyme leader peptidase (Kuhn and Wickner, 1985). For M13 procoat protein the mature region is additionally involved in all three steps. This shows that membrane insertion is a complex sequence of conformational events the entire protein has to go through.

Materials and methods

Bacterial strains and plasmids

E.coli K12 strains HJM114 [Δ*lacpro*, F'(*lacpro*)], UT580 [Δ(*lac-proAB*), *supD*, F'*lacI*^{q}, ZΔM15, *proA*$^+$*B*$^+$] and LC137 (*htpR*, *lon*, *lac*$_{am}$, *trp*$_{am}$, *pho*$_{am}$, *mal*$_{am}$, *rpsL*, *supC*^{ts}, *tsc*::Tn*10*) are from our laboratory collection. *E.coli* RZ1032 (*dut*, *ung*$^-$) has been described by Kunkel (1985). M13mp19 containing the wild-type procoat gene in its polylinker region was used for oligonucleotide-directed mutagenesis. As an expression vector, we used the pING-1 plasmid containing wild-type and mutant procoat genes under the control of the *araB* promoter (Kuhn and Wickner, 1985). Plasmid pSP65 (Melton *et al.*, 1984) was used for *in vitro* transcription.

Oligonucleotide-directed mutagenesis

For the construction of the deletion mutant ΔN30, the oligonucleotide 5'-CGGCTACAGAATTCATGAGG-3' was annealed to single stranded DNA of phage M13mp19 with the cloned procoat gene in its polylinker region. According to the method of Kunkel (1985) this phage had been propagated on *E.coli* RZ1032 to enhance the rate of mutagensis. *E.coli* DNA polymerase I (large fragment) and T4 DNA ligase (Boehringer) were used to prepare the covalently closed, double stranded plasmids. Following transfection into *E.coli* UT580 the replicative form of phage DNA of the different clones was prepared. The desired deletion was verified by screening for the presence of the introduced *Eco*RI restriction site and DNA sequencing. A 297 bp fragment containing the mutated procoat gene was isolated after digestion with *Sal*I and *Rsa*I, electrophoretic separation on a 1% agarose gel, and adsorption to DEAE-cellulose. This fragment was ligated with pING-2 vector previously cut by *Sal*I and *Sma*I. The pING-2 vector is a derivative of the pING-1 vector (Kuhn and Wickner, 1985) where the *Eco*RI site in the polylinker region was destroyed by a filling in reaction. For transformation, the method of Cohen *et al.* (1973) was used. All enzyme reactions and DNA preparations were performed as described by Maniatis *et al.* (1982).

For the construction of insertion mutants, equal amounts of two complementary oligonucleotides 5'-AATTCGCTTCGTCGTCGG-3' and 5'-AATTCCGACGACGAAGCG-3' were prehybridized at 86°C for 10 min resulting in a double stranded DNA fragment with *Eco*RI overhangs. Ligation of this fragment into *Eco*RI opened plasmids ΔN30 and ΔC30 resulted in either plasmids ΔN38 or ΔN39 and ΔC38 or ΔC39 depending on the orientation of the inserted DNA fragment. The plasmids were transformed into *E.coli* HJM114, ampicillin resistant colonies were grown and an analytical restriction digest of isolated plasmids with *Dde*I showed the insertion of the fragment. The orientation of the inserted fragment was analysed by DNA sequencing of the various plasmids. The same procedure with the oligonucleotides 5'-AATTTTTTAAAGCGCGCA-3' and 5'-AATTTGCGCGCTTTAAAA-3' was applied to construct plasmids ΔC36 and ΔC37.

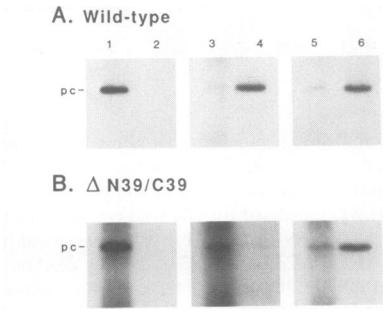

Fig. 7. Binding of newly synthesized procoat to liposomes. Translation of *in vitro* transcripts coding for wild-type procoat (**A**) and the mutant ΔN39/C39 (**B**) was carried out in a rabbit reticulocyte lysate supplemented with [^{35}S]methionine. The translation reaction was performed at 30°C for 60 min in the absence (lanes 1 and 2) and in the presence (lanes 3–6) of liposomes. The lipsomes made of 25% PG and 75% PC were added at 3 μg/ml (lanes 3 and 4); those made of 100% PC were added at the same concentration (lanes 5 and 6). The liposomes were separated from the translation reaction by floating through a sucrose step gradient. The bottom fractions (lanes 1, 3 and 5) and the top fractions (lanes 2, 4 and 6) of the respective gradients were analysed by SDS–PAGE and fluorography.

Cell fractionation

Cell fractionation experiments were performed following the procedure of Russel and Model (1982). 0.5 ml cultures of *E.coli* LC137 bearing plasmids with either wild-type or mutant procoats were grown at 30°C in M9 minimal medium containing 0.5% fructose to a density of 2 × 10^8 cells/ml. The cultures were induced by adding 0.5% L-arabinose. 15 min later the induced cells were pulse-labelled with 50 μCi [^{35}S]methionine for 1 min, 0.5 ml of ice-cold 0.2 N NaOH was added and the samples were vortexed vigorously. After lysis, 20% trichloroacetic acid (TCA) was added to one half of the sample. The second half of the sample was centrifuged for 30 min at 50 000 *g*, divided into supernatant and pellet fractions and precipitated with 20% TCA. All precipitates were collected by centrifugation and analysed for procoat and coat or araB and lipoprotein by immunoprecipitation, SDS–PAGE and fluorography (Ito *et al.*, 1980).

In vitro transcription and translation

*Sal*I–*Pst*I fragments from plasmids pQN8 and ΔN39/C39 encoding wild-type and the mutant procoat respectively, were cloned into plasmid pSP65 behind the SP6 promoter region. Transcription with SP6 polymerase (Biofinex) was carried out at 40°C for 45 min and the transcripts were immediately used for translation (Melton *et al.*, 1984; Krieg and Melton, 1984). Cell-free translation with rabbit reticulocyte lysate (Boehringer) was done for 60 min at 30°C in the presence of [^{35}S]methionine (Zimmermann and Mollay, 1986).

Binding assay of procoat protein to liposomes

Liposomes containing 75% phosphatidylcholine and 25% phosphatidylglycerol were prepared according to standard procedures (Racker, 1979).

The final lipid concentration was 16 μg/μl in 50 mM HEPES–KOH pH 7.4, 2 mM MgAc, 50 mM KAc, 1 mM DTT. Translation was carried out in the presence of 1 μg/μl liposomes. 13.5 μl of this translation reaction was placed in a Beckman airfuge tube, adjusted with 36.5 μl of 68% sucrose in HEPES–KOH pH 7.4, 2 mM MgAc, 50 mM KAc, 1 mM DTT to a final sucrose concentration of 50%, overlayed with 90 μl 17% sucrose in the same buffer and 40 μl buffer alone to form a step gradient. Following centrifugation in a Beckman airfuge (Rotor A-100/30) at 25 p.s.i. for 40 min the liposomes were floated to the top of the gradient. Fractions were collected by running out the gradient and were analysed by gel electrophoresis and fluorography. For a control, liposomes were labelled with [dioleoyl-1-^{14}C]-phosphatidylcholine (New England Nuclear). After centrifugation >90% of the labelled lipid was found in the top fraction.

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