

# Anionic phospholipids and protein translocation

Ben de Kruijff\*

*Department of Biochemistry of Membranes, Center for Biomembranes and Lipid Enzymology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*

Received 8 April 1994

## Abstract

Anionic phospholipids determine, in diverse ways, the membrane interaction of proteins involved in or undergoing membrane insertion or translocation. How these lipids modulate protein localization, organization, folding and membrane insertion is herein summarized and generalized, leading to a proposal for the function of anionic lipids in cellular transport of newly synthesized proteins.

**Key words:** Anionic phospholipid; Protein insertion and translocation; Protein folding; Lipid–protein interaction; Targeting signal

## 1. Introduction

Translocation of proteins into and across biological membranes is an obligatory step in the transport of many newly synthesized proteins and is essential for cellular processes such as secretion and membrane and organelle biogenesis. Cell killing also involves protein translocation processes by bacterial polypeptide toxins and the replication of viruses. In the last decade much progress has been made in establishing the main cellular protein transport routes and the identification of the components involved in the membrane insertion and translocation steps.

The purpose of this article is (i) to identify the current view of the involvement of anionic membrane lipids in protein translocation, and (ii) to specify and generalize the roles of lipids in membrane binding and insertion and polypeptide folding, common steps in all protein transport processes.

## 2. Evidence and arguments for the involvement of phospholipids in protein translocation: the specific roles for anionic lipids

Direct insight into the involvement of particular phospholipid classes for membrane translocation of proteins should come from studies in which the level of a particular lipid in a membrane is regulated and related to protein transport across that membrane. Such studies have been carried out successfully using phospholipid biosynthetic mutants in *E. coli* and revealed that anionic lipids are essential for efficient inner membrane insertion and

translocation of newly synthesized proteins following both sec-dependent [1–4] and -independent routes [5]. Accordingly, anionic lipid interactive compounds block translocation of precursor proteins which require these lipids for translocation and not of mutant proteins with modified signal sequences, allowing them to bypass the anionic lipid requirement [6,7].

Numerous studies in model systems ranging from complex reconstitution protein translocation systems composed of purified compounds [3] to advanced biophysical studies on more simple and defined model membranes [8–12] have given supportive evidence of the requirement of anionic lipids for membrane insertion and translocation of proteins in the prokaryotic secretion pathway. These studies also have given detailed mechanistic insights into the various ways the anionic lipids might fulfill their function in the process [13]. Given the similarities in the membrane translocation step in the pro- and eukaryotic secretion pathway, it can be anticipated that anionic lipids, which are also abundant components of endoplasmic reticulum membranes, fulfill similar functions in the latter pathway. It has also been clearly established that anionic lipids are important for membrane insertion of pore forming domains of polypeptide toxins [14,15].

Although conclusive experiments of the physiological situation are still missing, much circumstantial evidence has accumulated, suggesting that both in the general presequence-mediated import pathway of mitochondria [16], as well as in the specialized route followed by cytochrome *c* [17], anionic phospholipids are important for membrane insertion and translocation of mitochondrial precursor proteins. They appear to play complementary roles to those exerted by the many proteins which are directly or indirectly involved in protein transport [18].

Mechanistic insight into the process of protein import into peroxisomes and chloroplasts is still scarce, as is

\*Corresponding author. Fax: (31) (30) 522 478.

information on the role the membrane lipids play. Chloroplast protein import experiments with lipase-treated organelles [19], as well as studies using model membrane systems and precursor proteins [20], indicated that anionic lipids and glycolipids are involved in transport across the envelope membrane.

There are a number of theoretical aspects of membrane architecture worth considering when discussing the role of phospholipids in protein insertion and translocation. Firstly, lipids, by virtue of their amphipathic character, determine the barrier function of the membrane and, as such, are always directly or indirectly involved in membrane transport processes. During protein transport, the barrier should not be compromised. Lipids are structurally flexible membrane constituents and are ideally suited to anneal potential defects in the membrane generated by the insertion and translocation processes. Secondly, somewhere and sometime during insertion of (parts) of membrane proteins, they will have to come in to contact with the lipids. This applies also to the membrane-associated proteinaceous components of the translocation machinery. Thirdly, many of the targeting signals have amphipathic properties which make them very prone to interaction with the membrane lipids, and it appears that nature has designed these signals just for this property. If such interactions were not desired it would take effort from the system to avoid them.

### 3. How do anionic lipids influence and determine interactions between proteins and membranes?

Anionic lipids are present in all biological membranes and are often localized at the site of action in a biological process, the classical picture being the plasma membrane, where phosphatidylserine is normally confined to the inner leaflet where it fulfills its functions, for instance in signal transduction. Following activation of platelets, the molecule moves to the outer leaflet and then triggers the blood coagulation cascade.

One general idea emerging from such insights is that anionic lipids, by virtue of their charge, functionally localize and organize folded proteins and protein complexes by electrostatically interacting with positively charged domains on the protein(s). In protein translocation the anionic lipids could function similarly in the assembly of the translocation complex. In the prokaryotic secretion pathway, for instance, anionic lipids could correctly position the SecA dimer onto the membrane and the SecE and SecY proteins, thus enabling precursor proteins to insert into and move across the membrane. SecA is an ATPase which couples ATP hydrolysis to translocation. It is in functional interaction with many of the other components involved in translocation, including the precursor protein and the anionic

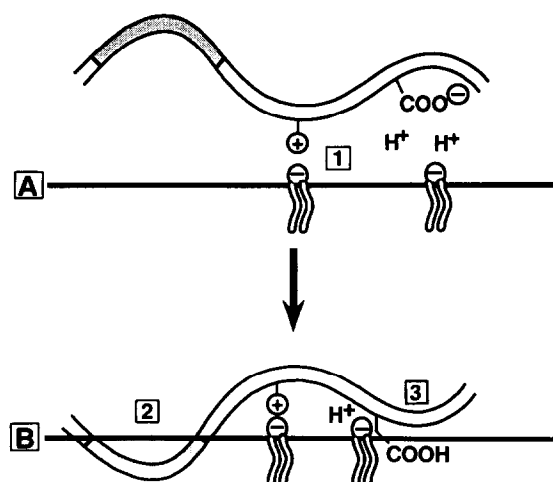


Fig. 1. Schematic representation of the ways in which anionic lipids mediate membrane binding and insertion of a polypeptide chain. Panel A shows part of an unstructured polypeptide chain containing a hydrophobic (shaded), a positive and a carboxylate-rich domain approaching a membrane containing anionic lipids, with an interfacial pH which is lower than the bulk pH. Panel B illustrates how the initial electrostatic interaction (1) results in membrane binding followed by insertion of the hydrophobic segment (2) and the protonated carboxylate-rich domain (3).

phospholipids [21]. Support for such suggestions comes from observations that ATP binding and hydrolysis affect the mode of SecA–lipid interaction [22], and that SecA has at least two lipid binding sites [23], one at the C-terminus involved in electrostatic interaction with anionic lipids, and one with a less-defined location involved in membrane insertion of the molecule. This leads, then, to a second general consequence of the interaction of anionic lipids in membranes with a polypeptide, namely that very often the initial electrostatic interaction (step 1 in Fig. 1) facilitates subsequent insertion of (part of) the polypeptide into the membrane. This could involve either hydrophobic stretches of the polypeptide (step 2) or stretches which are rich in aspartate and glutamate residues which become more readily protonated (and thus more hydrophobic) by the more acidic interfacial layer created by the accumulation of protons around the negatively charged lipid headgroups (step 3). Membrane insertion of such anionic polypeptide domains will depend on preceding other interactions which could involve receptor proteins or other membrane anchoring devices in order to overcome the unfavorable initial electrostatic repulsion. Considering the fact that SecA is an overall acidic protein, it is possible that interfacial protonation of acidic residues contributes to the anionic lipid-dependent membrane insertion of the protein.

Changes in polypeptide folding are intrinsically related to anionic lipid-mediated membrane insertion of proteins. Two aspects can be distinguished. Firstly, the interaction can result in destabilization of the tertiary

structure and (partial) unfolding. The degree to which this occurs will depend on the balance between the stability of the protein structure and the strength of the unfolding forces. Unfolding could be caused by the lower surface pH and/or the two-dimensional nature of the membrane in relation to the spatial distribution of the membrane-interactive domains on the protein. This, then, can result in molten globule states, such as in case of the pore-forming domain of colicin [15] or mitochondrial cytochrome *c* [28,31]. Anionic lipid-mediated unfolding of the presequence containing mitochondrial precursor proteins has been claimed to contribute to efficient protein import [24]. This hypothesis is attractive, because (i) proteins in general appear to move across membranes in an unfolded or more loosely folded state, (ii) the protein- to-lipid ratio of the mitochondrial outer membrane is low, and (iii) cardiolipin is present in the outer leaflet of the outer membrane [25]. The second consequence for the structure of (part of) a protein upon insertion into the lipid phase is that completely unstructured parts fold and acquire secondary structure (in particular  $\alpha$ -helices). Such folding behavior is especially relevant when considering targeting sequence–lipid interactions (see below), but also when analyzing the specialized import pathway followed by mitochondrial cytochrome *c*. This small and abundant intermembrane space-localized protein is synthesized without a cleavable presequence and is imported without the aid of cytosolically exposed receptors, does not require a membrane potential for import and by-passes the components of the putative general insertion protein/particle/pore [18]. Haemlyase, the enzyme localized in the intermembrane space, is responsible for covalent insertion of the haem group into the apoprotein during or shortly after outer membrane translocation, and is the only proteinaceous component so far shown to be essential for import [17]. Many model membrane studies have shown that interactions between anionic lipids and apocytochrome *c* give rise to unique properties of the system [26]. For instance, the ability of the N- terminus to spontaneously penetrate a bilayer up to the opposite interface without destroying the overall barrier function of the membrane [27] strongly suggests that the pathway followed by apocytochrome *c* across the outer membrane directly involves interactions between the precursor and the membrane lipids. Apocytochrome *c* lacks secondary structure in solution but becomes partly  $\alpha$ - helical upon interacting with the membrane lipids. These helices are located in the same regions as found in the folded holoprotein and are highly dynamic, both in terms of topology as well as in terms of stability [28–30]. The surprising and intriguing picture emerging is that, as a result of the anionic lipid-mediated membrane interaction, the holo- and apoprotein acquire a similar intermediate dynamic folding state [28,31] which could represent a translocation intermediate in the import process.

#### 4. Targeting signal–lipid interactions

In the prokaryotic secretion pathway, it became recently possible, via lipid biosynthetic mutants, to assess the importance of signal sequence–anionic lipid interactions in the functional process. This involved both the use of precursors with mutant signal sequences [6,7] as well as an analysis of the SecA-independent signal sequence-mediated translocation of the M13 procoat protein [5]. For other targeting signals the occurrence of such interactions in the functional process has not been directly studied, but many considerations and more indirect experimental observations suggest that such interactions occur. What, then, could be the function of targeting signal–anionic lipid interactions? Model membrane experiments suggest, within the presented general framework for protein–anionic lipid interactions, different possibilities within the themes of binding, insertion and folding.

The individual major classes of targeting signals will be addressed and a pictorial presentation of some of the possibilities is given in Fig. 2.

##### 4.1. Mitochondrial protein import

Matrix-targeting presequences are positively charged and the corresponding peptides have a high affinity for anionic lipids [16]. Due to the periodic distribution of the positively charged residues they most likely function as amphipathic helices, with one side of the helix positively charged and the other side hydrophobic. At which stage in the process this motif is adopted and possibly recognized is not known but it was shown that presequence peptides undergo a random coil-to-helix transition upon interacting with, and penetrating into, a lipid layer [16].

The following suggestions on the functionality of presequence–lipid interactions emerged from studies with model membranes: (i) the inserted  $\alpha$ -helix could function as a recognition motif for proteinaceous components of the translocation apparatus; (ii) the interaction could contribute to the efficiency of organelle-specific targeting because (a) two-dimensional surface diffusion to proteinaceous receptors in the outer membrane should be more efficient than three-dimensional diffusion via the cytosol and (b) a specific interaction occurs between the presequence [32,33] and cardiolipin, an anionic phospholipid in eukaryotic cells exclusively located in the mitochondrion and present in both the outer and inner membrane [36]; and (iii) the presequence–lipid interaction could bring the presequence into the electrical field over the inner membrane (inside negative) and thereby allow re-orientation of the presequence into or possibly even across that membrane [34,35]. The ability of presequence peptides to induce interbilayer contacts with a pronounced specificity for cardiolipin suggests that presequences bring about transient contacts between the outer and inner membrane and thereby hand over the presequence

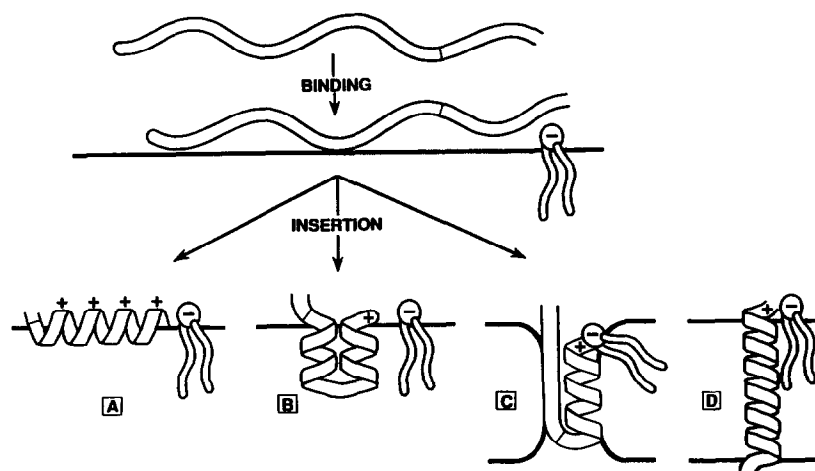


Fig. 2. Interactions between targeting signals and anionic lipids containing membranes. Electrostatic interactions between positively charged residues on the extension peptide and anionic lipids increase membrane binding, which is followed by insertion and induction of specific structural motifs, such as the amphipathic helix in the case of mitochondrial presequences (A), a looped (B) or extended (C)  $\alpha$ -helical conformation of signal sequences operating in the secretion pathway, and a membrane-spanning helical conformation of the transmembrane part of an integral membrane protein (D).

from the outer to the inner membrane [33]. At this point, it is worthwhile recalling that, within eukaryotic cells, only the mitochondrion experiences a potential across the inner membrane which could 'lock' the presequence into the organelle. The optional additional and often more hydrophobic intramitochondrial routing signals within the presequence could be involved in lipid interactions similarly as described below for signal sequences.

#### 4.2. Chloroplast protein import

The transit sequences functioning in the import of chloroplast proteins into the stroma are the most degenerated topogenic signals known so far [37]. They are large with a variable length and are essential and sufficient for import but lack virtually any primary structure homology, nor has a particular motif been identified. Most of their sequence is essential for chloroplast-specific import. The transit has no structure in solution, but upon interaction with an anionic lipid-containing membrane interface, secondary structure is induced [38]. This observation, together with the specificity of chloroplast-specific glycolipids for membrane insertion of the transit sequence [20], points to roles of transit sequence–lipid interactions in targeting and outer membrane insertion. H-bonding between the transit sequence, which is rich in hydroxylated amino acids, and the carbohydrate part of the glycolipids could contribute to unique chloroplast-specific recognition events.

#### 4.3. Prokaryotic secretion pathway

Signal peptide–lipid interactions have been the most extensively studied and the consequences of these interactions for both signal peptide and lipid structure are best understood [8]. Anionic lipids, via electrostatic interactions with the positive charge at the N-terminus of

the signal sequence, contribute to membrane binding and insertion of the peptide into the hydrophobic phase [39], which is accompanied by  $\alpha$ -helix formation [40]. The inserted form appears to be structurally flexible [10] and monolayer data suggest that it can attain both a conformation looped around the  $\alpha$ -helix breaking amino acid residue often present in signal sequences, and a more extended conformation [9]. Unlooping of the signal sequence would bring the signal sequence cleavage site, and thereby the N-terminus of the mature protein, to the opposite membrane interface [13]. A signal sequence is distinguishable from a transmembrane helical segment of a protein in the shorter length of the hydrophobic domain. In a transmembrane conformation, this will result in a mismatch in length of the signal sequence and the thickness of the bilayer, causing membrane dimpling. In lipid bilayers such a situation will result in the formation of type II non-bilayer lipid structures [41] which indeed have been observed for signal peptides [12]. Within the target biomembrane, the energetically unfavorable dimpling could result either in a shift in the conformational equilibrium to a looped conformation, or in lateral docking of the signal sequence onto a transmembrane proteinaceous translocation complex or onto the short transmembrane helix H2 of leader peptidase.

SecA–signal sequence interactions could facilitate membrane insertion of the signal sequence. The local perturbation of lipid organization (dimpling) upon insertion of the signal sequence could potentially facilitate the translocation of the more hydrophilic N-terminus of the mature protein. Also in the looped conformation the signal sequence could constitute a recognition motif, including possibilities for H-bonding within the membrane with proteinaceous components of the translocator. Consistent with the unlooping model of protein translo-

cation [13], fixing the signal sequence into a looped structure by disulfide crosslinking of strategically positioned cysteines arrests translocation at a very early stage [42].

#### 4.4. Transmembrane proteins

The targeting signal for an  $\alpha$ -helical segment of a transmembrane protein is the transmembrane domain itself. The positive-inside rule [43] states that a charge discrimination across transmembrane segments of proteins occurs in such a way that loops rich in positive charges remain in the cytoplasm whereas short loops, which are largely devoid of charges, are transferred across the membrane [44]. Anionic lipids present at the cytosolic face of the membrane could help to insert and anchor such transmembrane helices. Conversely, the arrangement of the transmembrane helices could form a topological determinant for the anionic lipids.

**Acknowledgements:** All present and past members of the Department of Biochemistry of Membranes involved in research on membrane insertion and translocation of proteins are thanked for their enthusiasm, skillful experiments, creative ideas and most enjoyable collaborations.

#### References

- [1] De Vrije, T., De Swart, R.L., Dowhan, W., Tommassen, J. and De Kruijff, B. (1988) *Nature* 334, 173–175.
- [2] Kusters, R., Dowhan, W. and De Kruijff, B. (1991) *J. Biol. Chem.* 266, 8659–8662.
- [3] Hendrick, J.P. and Wickner, W. (1991) *J. Biol. Chem.* 266, 24596–24600.
- [4] Cabelli, R.J., Dolan, K.M., Qcan, L. and Oliver, D.B. (1991) *J. Biol. Chem.* 266, 24420–24427.
- [5] Kusters, R., Breukink, E., Gallusser, A., Kuhn, A. and De Kruijff, B. (1994) *J. Biol. Chem.* 269, 1560–1563.
- [6] Phoenix, D.A., Kusters, R., Hikita, C., Mizushima, S. and De Kruijff, B. (1993) *J. Biol. Chem.* 268, 17069–17073.
- [7] Phoenix, D.A., De Wolf, F.A., Staffhorst, R.W.H.M., Hikita, C., Mizushima, S. and De Kruijff, B. (1993) *FEBS Lett.* 324, 113–116.
- [8] Gierasch, L.M. (1989) *Biochemistry* 28, 923–930.
- [9] Batenburg, A.M., Demel, R.A., Verkleij, A.J. and De Kruijff, B. (1988) *Biochemistry* 27, 5678–5685.
- [10] Wang, Z., Jones, J.D., Rizo, J. and Gierasch, L.M. (1993) *Biochemistry* 32, 13991–13999.
- [11] Keller, R.C.A., Killian, J.A. and De Kruijff, B. (1992) *Biochemistry* 31, 1672–1677.
- [12] Killian, J.A., De Jong, A.M.P., Bijvelt, J., Verkleij, J.A. and De Kruijff, B. (1990) *EMBO J.* 9, 815–819.
- [13] De Vrije, T., Batenburg, A.M., Killian, J.A. and De Kruijff, B. (1990) *Mol. Microbiol.* 4, 143–150.
- [14] Van der Goot, F.G., Didat, N., Pattus, F., Dowhan, W. and Letellier, L. (1993) *Eur. J. Biochem.* 213, 217–221.
- [15] Van der Goot, F.G., Gonzalez-Manas, J.M., Lakey, J.H. and Pattus, F. (1991) *Nature* 354, 408–410.
- [16] Tamm, L.K. (1991) *Biochim. Biophys. Acta* 1071, 123–148.
- [17] Stuart, R.A. and Neupert, W. (1990) *Biochimie* 72, 115–121.
- [18] Kiebler, M., Becker, K., Pfanner, N. and Neupert, W. (1993) *J. Membr. Biol.* 135, 1991–2007.
- [19] Kerber, B. and Soll, J. (1992) *FEBS Lett.* 306, 71–74.
- [20] Van 't Hof, R., Van Klompenburg, W., Pilon, M., Kozubek, A., De Korte-Kool, G., Demel, R.A., Weisbeek, P.J. and De Kruijff, B. (1993) *J. Biol. Chem.* 268, 4037–4042.
- [21] Lill, R., Dowhan, W. and Wickner, W. (1990) *Cell* 60, 271–280.
- [22] Breukink, E., Demel, R.A., De Korte-Kool, G. and De Kruijff, B. (1992) *Biochemistry* 31, 1119–1124.
- [23] Breukink, E., Keller, R.C.A. and De Kruijff, B. (1993) *FEBS Lett.* 331, 19–24.
- [24] Endo, T. and Schatz, G. (1988) *EMBO J.* 7, 1153–1158.
- [25] Hovius, R., Thijssen, J., Van der Linden, P., Nicolay, K. and De Kruijff, B. (1993) *FEBS Lett.* 330, 71–76.
- [26] Rietveld, A. and De Kruijff, B. (1986) *Biosci. Rep.* 6, 775–782.
- [27] Jordi, W., Li-Xin, Z., Pilon, M., Demel, R.A. and De Kruijff, B. (1989) *J. Biol. Chem.* 264, 2292–2301.
- [28] De Jongh, H.H.J., Killian, J.A. and De Kruijff, B. (1992) *Biochemistry* 31, 1636–1643.
- [29] De Jongh, H.H.J., Brasseur, R. and Killian, J.A. (1994) *Biochemistry* (submitted).
- [30] Snel, M.M.E., De Kruijff, B. and Marsh, D. (1994) *Biochemistry* (in press).
- [31] Spooner, P.J.R. and Watts, A. (1991) *Biochemistry* 30, 3880–3885.
- [32] Ou, W.-J., Ito, A., Umeda, M., Inoue, K. and Omura, T. (1988) *J. Biochem.* 103, 589–595.
- [33] Leenhouts, J.M., De Gier, J. and De Kruijff, B. (1993) *FEBS Lett.* 327, 172–176.
- [34] De Kroon, A.I.P.M., De Gier, J. and De Kruijff, B. (1991) *Biochim. Biophys. Acta* 1068, 111–124.
- [35] Maduke, M. and Roise, D. (1993) *Science* 260, 364–367.
- [36] Hovius, R., Lambrechts, H., Nicolay, K. and De Kruijff, B. (1990) *Biochim. Biophys. Acta* 1021, 217–226.
- [37] De Boer, A.D. and Weisbeek, P.J. (1991) *Biochim. Biophys. Acta* 1071, 221–253.
- [38] Horniak, L., Pilon, M., Van 't Hof, R. and De Kruijff, B. (1993) *FEBS Lett.* 334, 241–246.
- [39] Demel, R.A., Goormaghtigh, E. and De Kruijff, B. (1990) *Biochim. Biophys. Acta* 1027, 155–162.
- [40] Keller, R.C.A., Killian, J.A. and De Kruijff, B. (1992) *Biochemistry* 31, 1672–1677.
- [41] Killian, J.A. (1992) *Biochim. Biophys. Acta* 1113, 391–425.
- [42] Nouwen, N., Tommassen, J. and De Kruijff, B. (1994) *J. Biol. Chem.*, in press.
- [43] Von Heijne, G. (1988) *Biochim. Biophys. Acta* 947, 307–333.
- [44] Andersson, H., Bakker, E. and Von Heijne, G. (1992) *J. Biol. Chem.* 267, 1491–1495.