Presequence-Mediated Intermembrane Contact Formation and Lipid Flow. A Model Membrane Study†

Zsolt Török,* Rudy A. Demel, Johanna M. Leenhouts, and Ben de Kruijff

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

Received August 31, 1993; Revised Manuscript Received February 14, 1994®

ABSTRACT: The ability of a synthetic peptide corresponding to the presequence of cytochrome *c* oxidase subunit IV from yeast to cause intermembrane contacts was investigated using monolayer techniques. The presequence inserted efficiently into the monolayer with a specificity for the mitochondrial cardiolipin. In the inserted form, the peptide strongly promoted the formation of close contacts with large unilamellar lipid vesicles present in the subphase, a property which was also specific for cardiolipin. The contacts formed were stable and tight and resulted in the flow of lipids from the vesicles to the monolayer. These results led to new suggestions on the involvement of intermembrane contact formation in mitochondrial protein import and membrane biogenesis.

Nuclear-encoded mitochondrial proteins cope with two major problems. They have to be specifically recognized by mitochondria, and they have to find their final intramitochondrial location. Most of them are synthesized as larger precursors with a cleavable, 15–70 amino acid residue long, amino-terminal extension termed presequence. These presequences were shown to contain sufficient information to target an attached protein to mitochondria and to its final intramitochondrial location. No sequence homology has been found for presequences directing proteins to the same submitochondrial compartment. Presequences, targeting proteins to the mitochondrial matrix, are generally rich in positively charged amino acids and have the capability of forming amphiphilic structures in the presence of negatively charged lipid interfaces (Tamm, 1991). After being recognized, the precursors have to be translocated into or across one or two membranes with the help of the mitochondrial translocation machinery [for a review, see Glover and Lindsay (1992)]. Although the exact mechanism of the translocation is not known, it seems likely that it occurs through contact sites between the outer and inner mitochondrial membranes. It was shown that these contact sites are not static structures but their formation is dynamic (Jascur et al., 1992; Pfanner et al., 1992). It is believed that mitochondria contain two distinct translocation systems, one in the outer and one in the inner membrane (Rassow & Pfanner, 1991; Segui-Real et al., 1993). The inner membrane import machinery can completely translocate precursors across the membrane without involvement of the outer membrane machinery (Hwang et al., 1989). Translocation of the presequence of precursors across the inner membrane requires an electric potential (negative inside) across that membrane (Martin et al., 1991). The outer membrane import machinery can similarly function independently; however, it is not able to completely translocate precursors which have to be translocated into or across the inner membrane (Mayer et al., 1993). This observation also suggests the cooperative action of the two transport systems. It is an important question how the translocation contact sites are formed. The factors and the type of interactions involved in the formation of translocation contact sites are not known. Components of both membranes and/or the intermembrane space may initiate the interaction between the transport systems, but the participation of the transported precursor itself is also possible. The precursors may initiate the interaction either directly, for instance by interacting with inner membrane components (lipids, proteins) after emerging from the inside of the outer membrane, or indirectly, for instance by inducing structural rearrangements in the outer membrane.

The aim of the present study was to get insight into the possibility that interactions between the presequence and mitochondrial lipids can mediate close contacts between membranes. To tackle this question, a monolayer approach was chosen which can both qualitatively (surface pressure measurement at constant surface area) and quantitatively (surface radioactivity measurement) give insight into the formation of contacts between lipid monolayers and vesicles (Demel et al., 1989; Rojo et al., 1991). The two mitochondrial membranes were modeled by phospholipid monolayers and large unilamellar vesicles (LUVs),[1] and the well-studied presequence of cytochrome *c* oxidase subunit IV (p25) was used as a model peptide (Roise et al., 1986; Roise & Schatz, 1988; Tamm, 1991).

MATERIALS AND METHODS

Materials. The p25 peptide (H_3N^+-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu-COO^-) was prepared by solid-phase synthesis by D. Olshevski (University of California, San Diego) and purified as described (De Kroon et al., 1991).

† This study was supported by a long-term fellowship from the European Molecular Biology Organization (to Z.T.) and by the Foundation for Biophysics with financial aid from the Netherlands Organization for Scientific Research.

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; LUVs, large unilamellar vesicles; p25, presequence of cyclochrome *c* oxidase subunit IV; p*25, radioactivity labeled p25; BHCL, cardiolipin from bovine heart; ECCL, cardiolipin from *Escherichia coli*; CL, cardiolipin; eggPC, phosphatidylcholine from egg yolk; PC, phosphatidylcholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; PG, phosphatidylglycerol; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; PI, phosphatidylinositol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine.

0006-2960/94/0433-5589$04.50/0 © 1994 American Chemical Society

The identity of the peptide was confirmed by analysis of the amino acid composition and sequence. Stock solutions of the peptide in water were stored at −20 °C. p25 was labeled by iodo[1-^{14}C]acetamide (50 μCi, specific activity 58 mCi/mmol, Amersham) at its single cysteine residue as described (Tamm, 1986). Unreacted iodoacetamide was removed via gel filtration by centrifuging through Biogel P-2 packed in a 5-mL syringe. The specific activity of labeled p25 was 42 mCi/mmol. Peptide recovery was 70%. Cardiolipin from bovine heart (BHCL), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), and 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Egg yolk phosphatidylcholine (eggPC) was isolated and purified according to standard procedures. Cardiolipin from *Escherichia coli* (strain AH930) (ECCL) was a kind gift of Dr. J. A. Killian and was isolated and purified as described (Killian et al., 1994). Phosphatidylinositol (PI) from soybeans was obtained from Larodan Fine Chemicals AB (Malmö, Sweden). 1,2-Di[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (80 mCi/mmol) was obtained from Amersham.

Preparation of Large Unilamellar Vesicles. Dry lipid films were hydrated by vortexing in the same buffer as the subphase of the appropriate monolayer experiment. The dispersions were frozen and thawed 10 times and subsequently extruded 10 times through 400-nm pore-size polycarbonate filters as described (Hope et al., 1985). When it was desired, di[^{14}C]-oleoylphosphatidylcholine had been added to the lipids (maximum 1 mol %) before the dry lipid film was made.

Monolayer Experiments. All monolayer experiments were carried out in Teflon dishes at 25 °C in a thermostatically controlled box (Demel, 1982) essentially as described (Demel et al., 1989). The Teflon dish had a volume of 18.5 mL and a surface area of 29.6 cm^2. The presequence or vesicles were added to the subphase through a 0.5-cm^2 hole at an extended corner of the dish. The injected volumes were always <1% of the total subphase volume. The surface pressure was measured by the Wilhelmy method using a platinum plate. The amount of radiolabel at the interface was determined by a gas-flow detector (Demel, 1974) or by direct collection of the monolayer in a scintillation vial (Rietsch, 1977). The samples were counted and corrected for subphase-associated radioactivity by counting the same volume of subphase. Monomolecular lipid layers were spread from a $CHCl_3/CH_3OH$ (4:1 v/v) lipid solution to give the desired initial surface pressure on a subphase of 150 mM Na_2SO_4 (unless indicated otherwise), 20 mM HEPES (Sigma), and 2 mM EDTA (pH 7.0). The subphase was continuously stirred with a magnetic bar. The subphase was washed by injecting and ejecting buffer solution at opposite sides of the dish at a rate of 13 mL/min.

Other Methods. Protein was determined by the micro BCA assay (Pierce, Rockford, IL) using bovine serum albumin as a standard. Lipid phosphorus was determined according to Rouser et al. (1970). Concentrations of CL are always expressed on a phosphorus basis.

RESULTS

The approach we intended to follow was to first insert the presequence into a lipid monolayer (mimicking the outer mitochondrial membrane) and then to introduce large unilamellar vesicles into the subphase underneath the monolayer to study contact formation. This approach requires insight into the mode of insertion of p25 into the monolayer with special reference to possible specificities for the potential target lipids. The interaction of p25 with different phospholipids

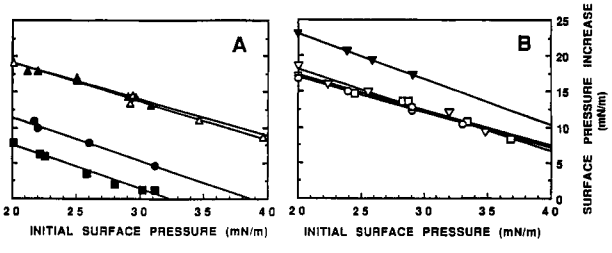

FIGURE 1: Surface pressure increase after injection of p25 underneath monolayers of different phospholipids at different initial pressures. The peptide concentration in the subphase was 1 μM. (A) BHCL (▲); ECCL (△); eggPC/BHCL (4:1) (●); eggPC (■) (B) DOPA (▼); DOPG (□); DOPS (▽); PI (○).

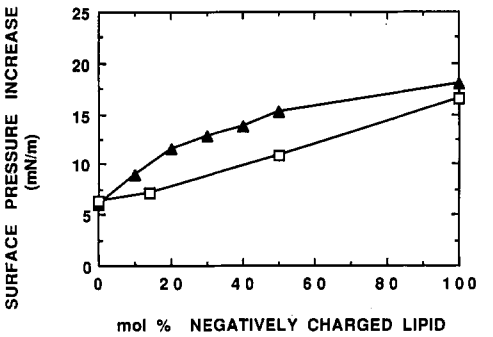

FIGURE 2: Monolayer surface pressure increase as a function of anionic lipid content. Monolayers of eggPC/BHCL (▲) and eggPC/DOPG (□) were formed at an initial surface pressure of 22 mN/m. The peptide concentration in the subphase was 1 μM.

was tested by measuring the peptide-induced surface pressure increase of lipid monolayers spread at the air–water interface up to different surface pressures (Figure 1). Extrapolation to high initial surface pressure allows the estimation of limiting insertion pressures (π_l) for the peptide, at which it is no longer able to insert into the monolayer. The peptide caused much larger surface pressure increases in the presence of CL from both bovine heart and *Escherichia coli* than in the case of the zwitterionic eggPC (Figure 1A). When the monolayer was made of an eggPC/BHCL (4:1) mixture which models the situation in mitochondria, the measured surface pressure changes were intermediate between the values for the two pure lipids. Insertion of p25 into monolayers of DOPG, DOPS, or PI resulted in somewhat lower, and into DOPA monolayers in higher, surface pressure increases compared to CL monolayers (Figure 1B). In mixed eggPC–anionic lipid monolayers, a specificity for mitochondrial cardiolipin over DOPG was observed (Figure 2). In the case of monolayers containing BHCL, the pressure increases were always higher than for DOPG and were higher than expected from the values anticipated from the behavior of the pure components assuming ideal behavior of the mixture.

Proteinase K was used to get insight into the localization of the presequence in the eggPC/BHCL (4:1) monolayer. The protease had no effect on the surface pressure of the peptide-free monolayer (result not shown). p25, preincubated with proteinase K, caused only a small increase in surface pressure (Figure 3, curve A), demonstrating that the intact presequence is required for monolayer insertion. If the proteinase K was added after the presequence had been able to interact with the monolayer, the surface pressure decreased quickly, which demonstrates that the peptide was accessible to proteinase K and is not completely shielded from the aqueous phase (Figure 3, curve B). The rate and extent of the proteinase K induced decrease in surface pressure showed a remarkable difference between CL and PG. Upon addition

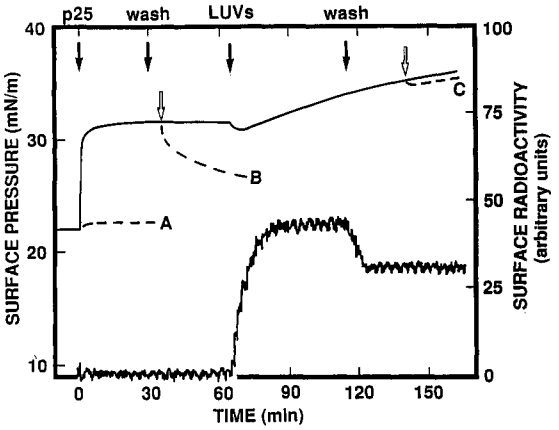

FIGURE 3: Intermembrane contact formation mediated by p25. The surface pressure (upper curve) and the surface radioactivity (lower curve) were recorded simultaneously during the p25-mediated interaction of ^{14}C-LUVs of eggPC/BHCL (4:1) with a monolayer of the same lipid mixture. The peptide concentration in the subphase after injection was 1 μM. The subphase was washed with peptide-free buffer at the indicated points. The concentration of the LUVs at the injection point was 27.5 μM (P_i). The dashed lines represent separate experiments in which (A) p25 was preincubated for 30 min at 25 °C with proteinase K (30 μg/mL) before it was added to the subphase or (B, C) at the open arrows proteinase K (30 μg/mL) was added to the subphase.

Table 1: Incorporation and Molecular Area of p*25 in Monolayers of BHCL and EggPC/BHCL (4:1)[a]

monolayer	expt	π_{p*25} (mN/m)	amount of p*25 in monolayer (pmol)	area per p*25 (Å^2/molecule)
eggPC/BHCL (4:1)	1	25.8	60	439
eggPC/BHCL (4:1)	2	25.3	57	414
BHCL	1	33.2	258	341
BHCL	2	33.0	280	312

[a] The monolayers were spread at an initial surface pressure of 22 mN/m. The peptide concentration at the injection point was 0.5 μM. The concentration of $p*25_{free}$ in the subphase after equilibration was determined to be 0.28–35 μM. π_{p*25}: surface pressure after incorporation of the peptide. The results of two independent experiments are shown.

of 30 μg/mL proteinase K at 25 °C, the times to reach a decrease in surface pressure, corresponding in absolute value to half of the presequence-induced pressure increase, were 11, 54, 1, and 16 min for PC/PG (4:1), PC/CL (4:1), PG, and CL, respectively (data not shown).

Insight into the molecular dimensions of p25 inserted into the monolayer could be obtained with presequence, radioactively labeled at its single cysteine residue (p*25). Although the labeled peptide caused a slightly less increase in surface pressure (compare Table 1 with Figure 1A), it is not expected (Tamm, 1986) that the labeling will affect the functional properties of the presequence. p*25, similarly to p25, caused a much higher surface pressure increase upon insertion into the CL monolayer, compared to the mixture of PC/CL (4:1) monolayer (Table 1). After the equilibrium pressure was reached, adsorbed and loosely bound peptide was eliminated by refreshing the subphase. Then the monolayer was collected, and the amount of p*25 associated was determined. From the amount of inserted peptide, it is possible to estimate the average area occupied by p*25 peptide in the monolayer knowing the area occupied by the lipids as a function of surface pressure (Tamm, 1986; Batenburg et al., 1988) and assuming additive molecular areas of the components. The estimated average molecular areas of p*25 were found to be approximately 430 and 330 Å^2 for the PC/CL (4:1) and CL system, respectively (Table 1).

The ability of p25 to cause intermembrane contacts was studied by following the interaction of vesicles with monolayers containing preinserted presequence. p25 was injected underneath an eggPC/BHCL (4:1) monolayer, causing the expected surface pressure increase (Figure 3). After equilibration of surface pressure, any free and loosely bound peptides were removed by washing the subphase. This washing procedure did not result in detectable changes in pressure, demonstrating a stable insertion of the presequence in the monolayer. Labeled LUVs of eggPC/BHCL (4:1) were then added to the subphase. This resulted in an initial decrease in surface pressure which is possibly due to the redistribution of some of the p25 from the monolayer to the vesicles. Immediately after the addition of the LUVs, the surface radioactivity rapidly increased. In the absence of p25, the vesicles did not cause significant pressure changes, and only a slightly increased surface radioactivity (<3 of the arbitrary units shown in Figure 3) could be observed, for which correction has been made in each case. Therefore, the immediate increase in surface radioactivity must reflect the association of the vesicles with the monolayer-bound peptide and thus clearly demonstrates the formation of intermembrane contacts by the presequence. Another interesting and novel phenomenon is revealed by this experiment. Five minutes after vesicle addition, the surface pressure started to increase slowly. This suggests that flow of lipids from LUVs to the monolayer takes place. The surface radioactivity reached an equilibrium value in 20 min. While the surface radioactivity remained at a constant level, that is, the number of bound vesicles did not change, the pressure was increasing continuously, suggesting that lipid flow continues to take place. Fifty minutes after vesicle addition, the subphase was washed with vesicle-free buffer, causing a drop in surface radioactivity by around 30% due to removal of loosely bound vesicles. However, the majority of the radioactivity was resistant to washing, demonstrating the stability of the intermembrane contacts. Since the surface pressure kept increasing with a similar rate, the lipids flowing to the monolayer originate from these tightly bound vesicles. Interestingly, the interaction of LUVs with the p25-containing monolayer made the peptide inaccessible to added proteinase K (Figure 3, curve C).

To analyze the lipid specificity of contact formation, experiments were also carried out with monolayers and labeled vesicles composed of a DOPG/eggPC (1:4) mixture. Contact formation was quantified by measuring monolayer-associated radioactivity after the subphase was washed and the monolayer was collected. The insertion of p25 resulted in binding of DOPG/eggPC (1:4) and BHCL/eggPC (1:4) vesicles equivalent to 0.46 ± 0.17 and 3.7 ± 0.6 nmol of phosphorus, respectively (data not shown). The much higher vesicle binding in the case of CL shows that contact formation is specific for vesicles containing this anionic lipid.

The ability of p25 to create close contacts between a phospholipid monolayer and LUVs was greatly increased with increasing salt concentration (Table 2); 300 mM Na_2SO_4 in the subphase resulted in a 20-fold increase in the amount of vesicles associated with the monolayer as compared with the experiment where no additional salt was present. The most likely explanation for this effect is that the increasing ionic strength of the subphase reduces the repulsive force between the negatively charged monolayer and vesicles and thereby promotes contact formation. The interaction between p25 and the negatively charged monolayer was also, but much less, influenced by the salt concentration (Table 2). The pressure increase after the addition of vesicles ($\Delta\pi_{LUVs}$),

Table 2: Effect of Ionic Strength on the Binding of ^{14}C-LUVs of EggPC/BHCL (4:1) to Monolayers of EggPC/BHCL (4:1) Containing p25[a]

$[Na_2SO_4]$ in subphase (mM)	$\Delta\pi_{p25}$ (mN/m)	$\Delta\pi_{LUVs}$ (mN/m)	vesicle binding (nmol of P_i)	covered area by LUVs (cm^2)
0	13.2	0.8	0.7	0.4
150	9.9	1.7	3.7	2.0
300	11.9	3.5	15.8	8.3

[a] $\Delta\pi_{p25}$, surface pressure increase induced by the peptide; $\Delta\pi_{LUVs}$, surface pressure change 40 min after the addition of LUVs. The amount of bound vesicles was determined 60 min after vesicle addition. The initial surface pressure was 22 mN/m. At their injection point, the concentration of the peptide was 1 μM, and that of the vesicles was 30 μM. The subphase contained the indicated amount of Na_2SO_4, 2 mM EDTA, and 20 mM HEPES (pH 7.0). The LUVs were formed in the same buffer as the subphase.

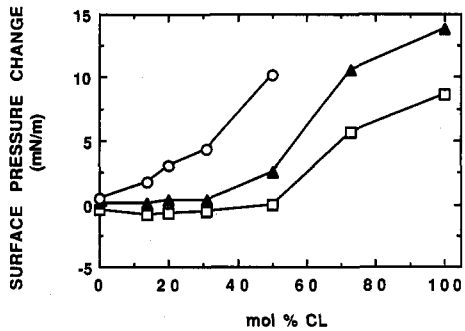

FIGURE 4: Effect of CL content of the vesicles on p25-induced contact formation. The monolayers of eggPC/BHCL (4:1) were spread at an initial pressure of 22 mN/m. p25 was injected into the subphase (1 μM). After equilibration, the subphase was refreshed with 7 volumes of peptide-free buffer. After washing, at time zero LUVs of eggPC/BHCL were injected into the subphase (25 μM P_i), and the surface pressure change was recorded after 5 (□), 20 (▲), and 60 (○) min.

corresponding to lipid flow, paralleled the amount of vesicles bound to the monolayer (Table 2).

To get more insight into the features of the contact-mediated lipid flow, we studied the role of the CL content of mixed PC/CL LUVs on the increase in surface pressure following contact formation. The lipid composition of the monolayer was the same in each experiment [CL/PC (1:4)] in order to maintain similar starting conditions (peptide binding and surface pressure) before vesicle addition. Figure 4 shows the surface pressure changes 5, 20, and 60 min after the addition of vesicles to the subphase. Increasing CL concentration in the vesicles eliminated the initial pressure decrease. Most pronounced was the higher rate of pressure increase with increasing CL concentration in the vesicles, suggesting increased lipid flow with increasing CL concentration in the trans-oriented membrane.

To follow the fate of the peptide during membrane contact formation, p*25 was injected under the monolayer, resulting in a simultaneous increase of pressure and surface radioactivity (Figure 5). After a maximum was reached in 5 min, the surface radioactivity decreased slightly, until, after 30 min, surface pressure and surface radioactivity reached an equilibrium. Then the subphase was refreshed by peptide-free buffer, resulting in a slight decrease in surface pressure, but in a large decrease of the surface radioactivity, demonstrating the presence of a considerable amount of loosely associated (noninserted) peptide. When subsequently unlabeled LUVs of eggPC/BHCL (4:1) were added to the subphase, the changes in surface pressure indicate that also in this case a contact was formed between the presequence in the monolayer

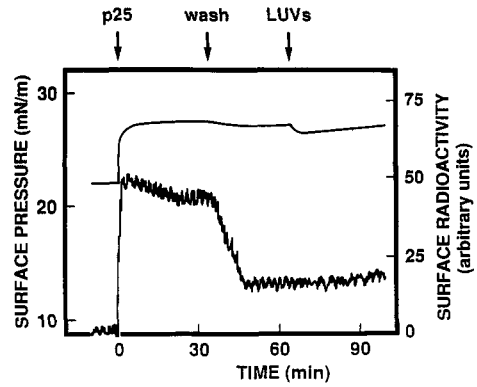

FIGURE 5: Interaction of LUVs with a monolayer containing ^{14}C-labeled p25. The lipid compositions of the vesicles and the monolayer were identical: eggPC/BHCL (4:1). The peptide concentration at the injection point was 0.5 μM. The subphase was washed with 7 volumes of peptide-free buffer at the indicated point. The concentration of LUVs was 25 μM (P_i). Surface pressure (upper curve); surface radioactivity (lower curve).

and the added vesicles, and thus that contact formation is due to the monolayer-inserted presequence. The constant surface radioactivity shows that the peptides remained associated with the interface during contact formation.

DISCUSSION

This study demonstrates that a mitochondrial presequence peptide (p25) can induce a close contact between two lipid–water interfaces. The monolayer–vesicle system used allows for a mechanistic understanding of contact formation because of the following features. First, the process could be studied in a sequential order mimicking the mitochondrial protein import process. The presequence peptide–monolayer interaction models the interaction between the presequence with the mitochondrial outer membrane. The vesicles in the subphase model the next biological target, i.e., the mitochondrial inner membrane. Second, both the behavior of the lipid monolayer (surface pressure) and contact formation (surface radioactivity in conjunction with radioactive LUVs) could be monitored continuously and simultaneously. Third, by making use of radiolabeled peptide and proteinase K, the fate of the peptide during the process of contact formation could be followed. Fourth, the obtained data allow for a quantitative analysis of the process.

One important result of our studies is the observed specificity of interaction between the presequence and cardiolipin. This was in particular revealed by the efficiency of insertion in mixed PC/CL monolayers, but also is indicated by the results on the mode of insertion. The presequence occupies a molecular area of approximately 430 Å^2 in the mixed PC/CL (4:1) system (our results), which is less than the value of 560 Å^2 reported by Tamm (1986), determined similarly in a PC/PG (4:1) mixed monolayer system. The presequence also appears to occupy a smaller molecular area in pure CL monolayers, 330 Å^2 (this study), as compared to pure PG monolayers, 530 Å^2 (Tamm, 1986). The most simple interpretation for this difference is that the presequence is more deeply inserted in the case of the CL system. The proteinase K accessibility experiments support this explanation. The putative α-helical part of the presequence (Endo et al., 1989; Tamm & Bartoldus, 1990) could for instance be inserted more parallel to the acyl chains in the case of CL (as suggested by IR experiments; Goormaghtigh et al., 1989) and more perpendicular in the case of PG as suggested by Tamm (1986).

The molecular basis for the CL specificity in the presequence–monolayer insertion is not known but appears to be a more general property of presequences since it was observed that the adrenodoxin precursor binds specifically to CL-containing liposomes (Ou et al., 1988). The unique chemical nature of the headgroup, with its two phosphates at a distance of <9 Å (inferred from molecular modeling), could form a unique recognition motive in the membrane–water interface possibly allowing simultaneous interaction with two positively charged amino acids facing the same side of an amphipathic helix.

CL is a unique mitochondrial lipid which occurs in both mitochondrial membranes (Hovius et al., 1990) and is enriched in an isolated mitochondrial contact site fraction (Simbeni et al., 1991). The presequence when inserted into the monolayer could cause a tight association between the monolayer and vesicles in the subphase. This contact formation also is greatly facilitated by mitochondrial cardiolipin. It is unlikely that contact formation is mediated by a presequence peptide aggregate adsorbed to the surface of the membrane because contact formation was still observed after extensive washing and removal of loosely associated peptide. Therefore, it is proposed that it is the inserted form of this peptide which mediates the contact. The molecular architecture of the contact site is not known but could be a local point semifusion intermediate stabilized by cardiolipin (Siegel, 1986), which is known for its tendency to adopt an organization in structures with concave water–lipid interfaces (Cullis & De Kruijff, 1979).

Quantitative analysis of the presequence-induced contact formation in conjunction with protease experiments suggests that in the case of the PC/CL (4:1) monolayer–vesicle system the presequence redistributes in the monolayer during the contact site formation process. In a typical experiment, 100 pmol of p25 is found inserted in the monolayer (data obtained for p*25), which causes the stable association of an amount of LUVs corresponding to 4 nmol of phospholipid. Assuming a spherical shape of the vesicles which were found to have an average diameter of 200 nm (data not shown), then it can be calculated that ±7% of the area of the monolayer is covered by vesicles (Table 2). This means that a large part of the monolayer is not covered with vesicles despite their presence as an excess in the subphase. The presequence remains largely associated with the monolayer during contact formation as inferred from surface radioactivity and surface pressure measurements. Because proteinase K cannot degrade the presequence any more under these conditions, it has to be concluded that the presequence is concentrating by lateral diffusion in the monolayer–vesicle contact area. Rapid diffusion of the presequence in the monolayer has been described (Frey & Tamm, 1990).

Subsequent to contact formation in the PC/CL (4:1) monolayer–vesicle system, the surface pressure of the monolayer increased largely and for prolonged times. The extent of this effect increased with increasing CL concentration in the vesicles (Figure 4). We interpret this result in terms of lipid transfer from the LUVs to the monolayer as a result of presequence-induced monolayer–vesicle contacts. Our interpretation is based on the fact that the subphase was washed with protein-free buffer before the addition of vesicles. After equilibration, the surface radioactivity remains at a constant level in the case of both p*25 and labeled LUVs (Figures 3 and 5), that is, neither additional peptide insertion nor further vesicle binding could occur. After removal of the free and loosely associated vesicles, the pressure increase continues with a similar rate as before the washing, which means that the effect is caused by the bound vesicles. In a typical experiment, the increase in surface pressure after 60 min corresponds to a flow of 2.5–5% of the lipids of the vesicles to the monolayer. It is of interest to compare the present findings with a recent study in which it was reported that presequence addition to LUVs results in cardiolipin-specific increases in turbidity which were interpreted as vesicle aggregation and which could be reversed upon applying a transmembrane potential (Leenhouts et al., 1993). Also, in this vesicle system lipid flow could be measured with fluorescence techniques. Employing BHCL/DOPC (3:7) large unilamellar vesicles, with and without fluorescently labeled phospholipids, such as pyrene-PC or the energy-transfer couple NBD-PE/Rhodamine-PE (Tournois et al., 1990; Struck et al., 1981), 15 min after presequence addition to a mixture of labeled and unlabeled vesicles lipids of the different vesicles were mixed for 40–50% (J. M. Leenhouts, unpublished observations). These observations support the view of a very close contact between the two opposing lipid layers and are consistent with a presequence-induced semifusion intermediate in which the opposing monolayers can mix their lipids. It should be stressed that for a number of other proteins, apocytochrome *c* (Demel et al., 1989), creatine kinase (Rojo et al., 1991), and SecA (Breukink et al., 1993), which efficiently created monolayer–vesicle contacts, no lipid flow was observed. Interestingly, the lung surfactant protein Sp-C did cause lipid flow in such systems (Oosterlaken-Dijksterhuis et al., 1991), which is suggested to be of physiological significance, because surfactant formation and function require massive lipid flow.

Speculation from the present results would be that presequence-mediated intermembrane contacts could be involved in lipid flow between the outer and inner mitochondrial membranes. Lipid flow does occur between the two membranes, but the process(es) by which it occurs is(are) largely unknown. A coupling between protein import and lipid flow could be a direct way of ensuring concerted membrane and organelle biogenesis. Whether presequences are involved in contact formation in mitochondria is not known. The present and a previous study (Leenhouts et al., 1993) offer a simple mechanism whereby such contacts can be transiently formed. The presequence emerging at the inside of the outer membrane could create a contact with the inner membrane involving cardiolipin. The presequence is now brought into the region where it can be sensed by the membrane potential across the inner membrane which electrophoretically moves the presequence across that membrane.

ACKNOWLEDGMENT

We thank Mrs. Gerda de Korte-Kool for purifying the synthetic presequence.

REFERENCES

Batenburg, A. M., Demel, R. A., Verkleij, A. J., & De Kruijff, B. (1988) *Biochemistry 27*, 5678–5685.

Breukink, E., Keller, R. C. A., & De Kruijff, B. (1993) *FEBS Lett. 331*, 19–24.

Cullis, P. R., & De Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.

De Kroon, A. I. P. M., De Gier, J., & De Kruijff, B. (1991) *Biochim. Biophys. Acta 1068*, 111–124.

Demel, R. A. (1974) *Methods Enzymol. 32*, 539–545.

Demel, R. A. (1982) in *Membranes and Transport* (Martonosi, A. N., Ed.) Vol. 1, pp 159–164, Plenum Publishing Co., New York.

Demel, R. A., Jordi, W., Lambrechts, H., Van Damme, H., Hovius, R., & De Kruijff, B. (1989) *J. Biol. Chem. 264*, 3988–3997.

Endo, T., Shinada, F., Roise, D., & Inagaki, R. (1989) *J. Biochem. 106*, 396–400.

Frey, S., & Tamm, L. K. (1990) *Biochem. J. 272*, 713–719.

Glover, L. A., & Lindsay, J. G. (1992) *Biochem. J. 284*, 609–620.

Goormaghtigh, E., Martin, I., Vandenbranden, M., Brasseur, R., & Ruysschaert, J. M. (1989) *Biochem. Biophys. Res. Commun. 158*, 610–616.

Hope, M. J., Bally, M. B., Webb, G., & Cullis P. R. (1985) *Biochim. Biophys. Acta 812*, 55–65.

Hovius, R., Lambrechts, H., Nicolay, K., & De Kruijff, B. (1990) *Biochim. Biophys. Acta 1021*, 217–226.

Hwang, S., Jascur, T., Vestweber, D., Pon, L., & Schatz, G. (1989) *J. Cell Biol. 109*, 487–493.

Jascur, T., Goldenberg, D. P., Vestweber, D., & Schatz, G. (1992) *J. Biol. Chem. 267*, 13636–13641.

Killian, J. A., Koorengevel, M. C., Bouwstra, J. A., Gooris, G., Dowhan, W., & De Kruijff, B. (1994) *Biochim. Biophys. Acta 1189*, 225–232.

Leenhouts, J. M., De Gier, J., & De Kruijff, B. (1993) *FEBS Lett. 327*, 172–176.

Maduke, M., & Roise, D. (1993) *Science 260*, 364–367.

Martin, J., Mahlke, K., & Pfanner, N. (1991) *J. Biol. Chem. 266*, 18051–18057.

Mayer, A., Lill, R., & Neupert, W. (1993) *J. Cell Biol. 121*, 1233–1243.

Oosterlaken-Dijksterhuis, M. A., Haagsman, H. P., Van Golde, L. M. G., & Demel, R. A. (1991) *Biochemistry 30*, 10965–10971.

Ou, W. J., Ito, A., Umeda, M., Inoue, K., & Omura, T. (1988) *J. Biochem. 103*, 589–595.

Pfanner, N., Rassow, J., Van Der Klei, I. J., & Neupert, W. (1992) *Cell 68*, 999–1002.

Rassow, J., & Pfanner, N. (1991) *FEBS Lett. 293*, 85–88.

Rietsch, J., Pattus, F., Desnuelle, P., & Verger, R. (1977) *J. Biol. Chem. 252*, 4313–4318.

Roise, D., & Schatz, G. (1988) *J. Biol. Chem. 263*, 4509–4511.

Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., & Schatz, G. (1986) *EMBO J. 5*, 1327–1334.

Rojo, M., Hovius, R., Demel, R. A., Nicolay, K., & Wallimann, T. (1991) *J. Biol. Chem. 266*, 20290–20295.

Rouser, G., Fleischer, S., & Yamamoto, A. (1970) *Lipids 5*, 494–496.

Segui-Real, B., Kispal, G., Lill, R., & Neupert, W. (1993) *EMBO J. 12*, 2211–2218.

Siegel, D. P. (1986) *Biophys. J. 49*, 1171–1183.

Simbeni, R., Pon, L., Zinser, E., Paltauf, F., & Daum, G. (1991) *J. Biol. Chem. 266*, 10047–10049.

Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.

Tamm, L. K. (1986) *Biochemistry 25*, 7470–7476.

Tamm, L. K. (1991) *Biochim. Biophys. Acta 1071*, 123–148.

Tamm, L. K., & Bartoldus, I. (1990) *FEBS Lett. 272*, 29–33.

Tournois, H., Fabrie, C. H. J. P., Burger, K. N. J., Mandersloot, J., Hilgers, P., Van Dalen, H., De Gier, J., & De Kruijff, B. (1990) *Biochemistry 29*, 8297–8307.