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Incorporation of a Synthetic Mitochondrial Signal Peptide into Charged and Uncharged Phospholipid Monolayers

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ABSTRACT: The interaction of the chemically synthesized 25-residue signal peptide of subunit IV of yeast cytochrome *c* oxidase with synthetic and natural phospholipids was studied by using a monolayer technique. Incorporation of the peptide into phospholipid monolayers was measured as surface area increase at constant surface pressure. The peptide was readily soluble in aqueous buffer, yet spontaneously inserted from an aqueous subphase into phospholipid monolayers up to limiting pressures of 30–40 mN/m. The incorporation of the positively charged peptide was strongly enhanced by the presence of negatively charged phospholipids. The molecular area of the signal peptide in monolayers was determined with a ^{14}C-labeled signal peptide and was 560 ± 170 Å^2. This is consistent with a 25-residue α-helical peptide incorporating with its long axis parallel to the plane of the monolayer. Incorporation isotherms into synthetic phosphatidylcholine and phosphatidylglycerol monolayers at different charge densities were analyzed in terms of a simple incorporation/binding model, involving (i) partitioning of the peptide into the monolayer and (ii) an in-plane binding reaction of the negatively charged phospholipids to the partitioned peptide.

In eucaroytes most proteins are encoded by the nucleus and translated on ribosomes in the cytoplasm. Many of them have to cross one or more membranes before they reach their final location inside or outside the cell. These include all secreted proteins and quite a large number of the polypeptides of chloroplasts and mitochondria. It has been recognized recently that N-terminal extensions ("signal", "leader", "transit" or "pre-" sequences) are responsible for intracellular sorting and the membrane translocation of these proteins. Most of them are synthesized as larger precursors, and the "signal" sequences are subsequently removed on the "trans" side of the membrane by specific proteases [for a review, see Wickner & Lodish (1985)]. One of the most intriguing questions relating to this process is how a polypeptide can select the correct target

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membrane from the large number of membranes exposed to the cytoplasm. Only in the last few years we have begun to understand some of the crucial features of membrane recognition and translocation in more detail: (i) common structural patterns have been found in mitochondrial signal peptides (von Heijne, 1986) and in chloroplast transit peptides (Karlin-Neuman & Tobin, 1986), (ii) specific receptors for signal peptides have been identified and characterized in the endoplasmic reticulum (Meyer et al., 1982; Walter et al., 1984) but not so far in mitochondria and chloroplasts, and (iii) several laboratories showed that presequences of several eucaryotic secretory and imported mitochondrial and chloroplast proteins are sufficient to transport attached foreign "passenger" proteins to and across the appropriate target membranes (Lingappa et al., 1984; Hurt et al., 1984; Horwich et al., 1985; van den Broeck et al., 1985).

Even if many of the components determining the high selectivity of membrane targeting have been identified in recent years, presently practically nothing is known about the mechanism of protein translocation across these membranes. However, recently signal peptides of several proteins, namely, the Lam B gene product of *Escherichia coli*, the bacteriophage M13 protein, and subunit IV of yeast cytochrome *c* oxidase, have been chemically synthesized in sufficient quantities for biophysical model studies (Briggs & Gierasch, 1984; Shinnar & Kaiser, 1984; Roise et al., 1986). Also, synthetic model peptides with repeating units of hydrophobic and charged amino acids were effective inhibitors of protein import into mitochondria (Ho et al., 1985). The subunit IV signal peptide comprises 25 amino acid residues, including 4 arginines and 1 lysine. Circular dichroism measurements have shown that its conformation is that of a random coil in an aqueous environment and largely α-helical in the presence of sodium dodecyl sulfate micelles. When arranged in an α-helix, most hydrophobic residues are located on one face of the helix opposite to most hydrophilic and charged residues, yielding an "amphiphilic" α-helix. It has been demonstrated that this peptide penetrates into mitochondrial lipid monolayers (at constant surface area) and that it facilitates the release of a trapped dye from lipid vesicles. The release of trapped dye is sensitive to the membrane potential and is enhanced when the potential is negative inside (Roise et al., 1986). In the present work, I wish to address the question of the specificity of this mitochondrial signal peptide for different lipids, using a quantitative monolayer technique. The monolayer area increase at constant surface pressure is measured with different natural and synthetic lipids at various peptide concentrations. Incorporation isotherms are obtained that can be analyzed in terms of hydrophobic and electrostatic components of the lipid–peptide interaction. In addition, monolayer experiments with isotopically labeled signal peptide yield the average molecular area per peptide and the lipid-to-peptide stoichiometries in the monolayer.

Materials and Methods

Materials. The peptide p25[1] was synthesized in the laboratory of J. H. Richards at the California Institute of Technology as described (Roise et al., 1986) and was a kind gift of Dr. G. Schatz (University of Basel). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were obtained from Avanti Polar Lipids, Inc. (USA), and bovine brain phosphatidylserine was obtained from Serva (FRG). They were pure on silica gel TLC and used without further purification. [^{14}C]Iodoacetamide was from New England Nuclear (USA) and had a specific activity of 24.4 mCi/mmol.

Mitochondrial Lipids. Total lipids were extracted from yeast mitochondria (strain D273-10B) by the method of Bligh and Dyer (1959). Phospholipids were prepared from the total lipid extract by precipitation from ice-cold acetone. Silica gel TLC plates were developed in $CHCl_3$–MeOH–H_2O (65:25:4) and revealed the expected mixture and relative amounts of the various lipids (Daum, 1985).

Peptide Concentrations. Concentrations of peptide solutions were determined by quantitative amino acid analysis. Absolute concentration values were converted into absorbance values obtained by the method of Lowry et al. (1951). This method was then used for routine experiments.

Reductive Alkylations. For labeling of p25 at its single Cys residue with [^{14}C]iodoacetamide, 140 nmol of p25 (molecular weight 2814) was dissolved in 0.1 M ammonium acetate, pH 8.1, to give a 1 mg/mL solution. The peptide was reduced with 70 nmol of dithiothreitol at 37 °C under argon for 1 h. This solution was added to 840 nmol of [^{14}C]iodoacetamide (3-fold excess over total sulfhydryl groups). The reaction mixture was kept under argon and incubated at 37 °C for 1–1.5 h. Unreacted iodoacetamide was removed by gel filtration on Biogel P-2 in a 5-mL syringe with centrifugation. The specific activities of labeled p25 varied in two experiments from 15 to 24 mCi/mmol, yielding labeling efficiencies of 62% and 98%, respectively. Peptide recovery was about 60% in both experiments.

Lipid Monolayers. All monolayer experiments were carried out in a circular Teflon trough with eight compartments (Model RMC2-T; Mayer Feintechnik, Göttingen, FRG). This trough is described in full detail by Fromherz (1975). Surface pressures were measured by the Wilhelmy method using plates cut from Whatman No. 1 filter paper and rinsed with methanol prior to use. Ion-exchanged and glass-distilled water was used to prepare all solutions and buffers. For most experiments the following buffer was used: 10 mM NaP_i, pH 7.0, 0.1 M NaCl, and 2 mM EDTA. Incorporation experiments were carried out as follows: two or three compartments of the trough were each filled with 25 mL of buffer, without connecting them. The surface area between the two Teflon barriers was reduced and cleaned by aspiration. A monolayer of the same lipid composition as used later was spread and removed in order to further clean the surface. After readjusting the surface area to 50 cm^2 the lipids were spread from hexane–ethanol (9:1) to yield a monolayer with a surface pressure of 12–14 mN/m. After 5 min the film was slowly compressed to the desired surface pressure. The instrument was then switched to the constant-pressure mode, and the change in surface area was recorded as a function of time. The desired amount of peptide (60 μM stock solution in water) was then injected with a Hamilton syringe into the monolayer subphase, which was stirred with a small magnetic stirring bar. After each experiment the trough was thoroughly cleaned with ethanol and distilled water. All measurements were done at ambient temperature (21 ± 2 °C).

Surface Radioactivity Measurements. Monolayer films containing ^{14}C-labeled peptide were collected through a short glass capillary by aspiration directly into 20-mL scintillation

[1] Abbreviations: BSA, bovine serum albumin; DLPG, 1,2-dilinoleoyl-*sn*-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-3-phosphoglycerol; EDTA, ethylenediaminetetraacetic acid; p25, 25-residue signal peptide of yeast cytochrome *c* oxidase subunit IV; P_i, inorganic phosphate; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; TLC, thin-layer chromatography.

$NH_3^{\oplus}$-met-leu-ser-leu-$arg^{\oplus}$-gln-ser-ile-$arg^{\oplus}$-phe-phe-$lys^{\oplus}$
pro-ala-thr-$arg^{\oplus}$-thr-leu-cys-ser-ser-$arg^{\oplus}$-tyr-leu-leu-$CONH_2$

FIGURE 1: Amino acid sequence of the synthetic signal peptide p25 of subunit IV of yeast cytochrome *c* oxidase. Hydrophobic amino acids are underlined, and charges are as indicated.

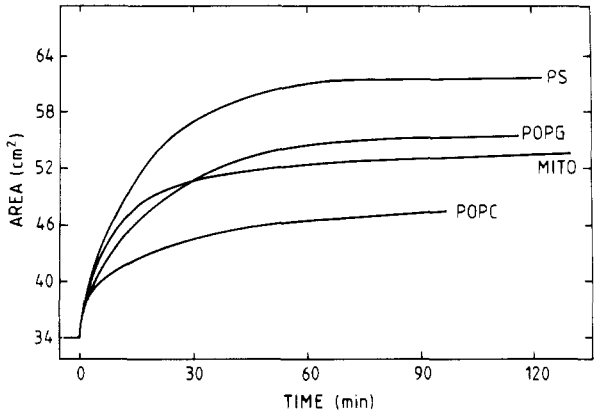

FIGURE 2: Interaction of the signal peptide p25 with phospholipid monolayers of different composition. The monolayer area increase due to peptide incorporation is measured at a constant monolayer surface pressure of 21 mN/m. Peptide (0.12 μM) was injected into the subphase (10 mM NaP_i, pH 7.0, 0.1 M NaCl, 2 mM EDTA) at time 0. MITO, yeast mitochondrial lipids.

vials. The glass capillary was prewashed with a 1% BSA solution. The monolayer was removed at constant surface pressure while decreasing the area. Typically, 40–50 cm^2 monolayers were collected in a volume of 0.4–0.8 mL. The samples were counted after addition of 19 mL of aqueous scintillation fluid. One-milliliter aliquots were also taken from the subphase, counted, and used to correct for subphase-associated counts in the monolayer samples. The monolayer counts were corrected for the total monolayer area and divided by the area increase due to peptide incorporation.

Results

The precursor of subunit IV of yeast cytochrome *c* oxidase contains 25 extra residues at the N-terminus that are proteolytically removed upon import into the inner mitochondrial membrane. A 25-residue peptide (p25), which exactly represents the presequence, has been synthesized (Roise et al., 1986), and its chemical structure is shown in Figure 1. The peptide is highly soluble in water (>20 mg/mL), but also very surface-active. In order to measure its surface activity, p25 was injected into 25 mL of 10 mM NaP_i buffer, pH 7.0, 0.1 M NaCl, and 2 mM EDTA (phosphate-buffered saline) with 40 cm^2 of a clean air–water interface available to give final peptide concentrations of 0.12–0.25 μM. The surface pressure increased very rapidly to about 13 mN/m and reached a final value of 17–18 mN/m within 40–60 min. All further experiments with lipid monolayers were carried out at surface pressures >20 mN/m in order to avoid nonspecific surface adsorption.

The interaction of p25 with different phospholipids and phospholipid mixtures was tested in a monolayer experiment shown in Figure 2. Monolayers were spread at 21 mN/m on 25 mL of phosphate-buffered saline, and the peptide was injected at time 0 to give 0.12 μM p25. Due to peptide incorporation the monolayer area increased with time and reached a plateau after about 60–90 min. The peptide interacted with different phospholipids selectively, and under identical conditions the largest amount of peptide was found to insert into phosphatidylserine monolayers and the lowest

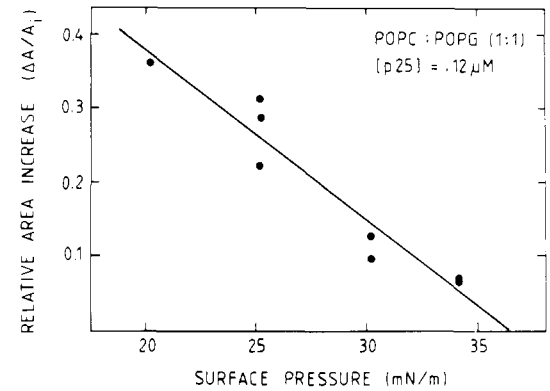

FIGURE 3: Incorporation of the signal peptide p25 into POPC–POPG (1:1) monolayers at different surface pressures. p25 incorporates up to a limiting surface pressure of 36.4 mN/m. The peptide concentration is 0.12 μM. Subphase: 10 mM NaP_i, pH 7.0, 0.1 M NaCl, 2 mM EDTA.

amount into POPC monolayers. The relative area increases ($\Delta A/A_i \times 100$) averaged from several experiments were 84.5% for phosphatidylserine from bovine brain, 67.4% for POPG, 63.0% for the mitochondrial lipid extract, a d 40.0% for POPC. The reproducibility of these final values was good (standard deviations 0–9%), but the kinetics of incorporation varied significantly in different experiments, probably due to the relatively slow mixing rates in the subphase.

A phospholipid bilayer or real biological membrane is probably best modeled with a monolayer at a surface pressure of about 25–34 mN/m (Demel et al., 1975; Schindler, 1979). In order to tesι whether peptide incorporation also occurs at higher monolayer pressures, the relative area increase was measured as a function of the monolayer surface pressure (Figure 3). When p25 was injected to give 0.12 μM under a monolayer of 1:1 (mol/mol) POPG and POPC, the relative area increase decreased linearly with the surface pressure, down to a limiting pressure of 36.4 mN/m, where no further area increase was observed. For all following experiments a surface pressure of 25 mN/m was chosen, which is sufficiently close to the bilayer "equivalence pressure" and still produces a large $\Delta A/A_i$ signal.

The results of Figure 2 demonstrate that monolayers of synthetic phospholipids can be as effective substrates for incorporation of the signal peptide as monolayers of the natural phospholipid mixture extracted from mitochondria. They also suggest that the surface charge density on the membrane could play an important role in modulating the strength of the lipid–peptide interaction. In order to investigate the influence of negatively charged phospholipids on the interaction, the relative monolayer area increase was measured for two synthetic phospholipids with identical fatty acyl chain composition, namely, POPC and POPG, as a function of peptide concentration in the subsolution. Incorporation into monolayers of the two lipids alone and in a 4:1 (mol/mol) mixture was measured (Figure 4). The results can be summarized as follows: (1) below a critical peptide concentration of approximately 3.75×10^{-8} M no area increase was observed; (2) the relative increase in POPC monolayers increased only very little from 0 to 0.5 μM total peptide concentration; (3) a strong relative area increase was observed in the presence of a POPG monolayer and the incorporation isotherm increased nonlinearly but monotonically in the measured concentration range; (4) no saturation was observed at the highest peptide concentrations investigated (0.55 μM); and (5) the incorporation isotherm for the POPC–POPG (4:1) monolayer falls in between those of the pure POPG and POPC monolayers.

Table I: Incorporation and Molecular Area of [^{14}C]Acetamide-Labeled p25 in Negatively Charged Phospholipid Monolayers at 25 mN/m

total concn of p25 (μM)	free concn of p25 (μM)	increase in monolayer area (cm^2)	amount of p25 in monolayer (pmol)	area per p25 (Å^2/molecule)
POPG monolayer, 25 mN/m				
0.12	0.033	14.5	743	324
0.12	0.066	16.3	580	467
0.24	0.097	21.3	439	806
0.24	0.120	19.2	624	511
POPC–POPG (4:1) monolayer, 25 mN/m				
0.05	0.021	2.8	78	593
0.12	0.078	6.7	168	661
average area per p25				561 ± 167

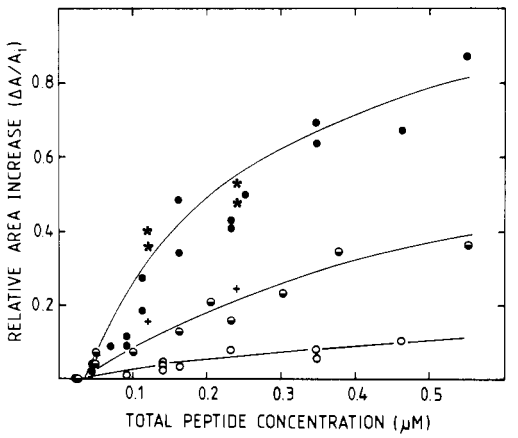

FIGURE 4: Incorporation isotherms of the signal peptide p25 into pure POPG (●), 4:1 POPC–POPG (⊖), and pure POPC (○) monolayers at 25 mN/m. Data obtained under identical conditions, but with [^{14}C]acetamide-labeled p25 are also included (*) POPG monolayer, and (+) 4:1 POPC–POPG monolayer. Subphase: 10 mM NaP_i, pH 7.0, 0.1 M NaCl, 2 mM EDTA.

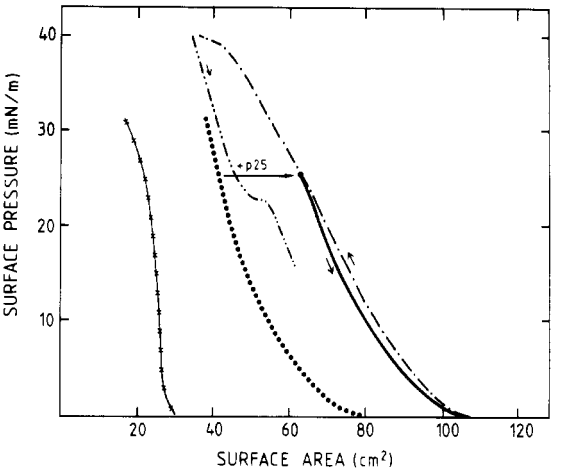

FIGURE 5: Pressure–area isotherms of POPG (dotted line) and POPG/p25 monolayers (solid and dashed lines). p25 was incorporated into the POPG monolayer at 25 mN/m and 0.25 μM total concentration. The resultant pressure–area isotherm shows hysteresis [(—) decompression, (-·-) compression, (-··-) decompression from 40 mN/m]. The crossed line represents a calculated difference curve POPG/p25 minus POPG. The subphase was 10 mM NaP_i, pH 7.0, 0.1 M NaCl, and 2 mM EDTA, and the rate of compression and decompression was 5 cm^2/min.

In order to interpret these incorporation isotherms further, it was necessary to know the average area occupied by a signal peptide molecule in the monolayer. This can be obtained from measuring the surface concentration of the peptide. p25 was labeled at cysteine-19 with [^{14}C]iodoacetamide to a specific activity of 15–24 mCi/mmol. The labeled peptide incorporated into POPG and POPC–POPG (4:1) monolayers with the same efficiency as the unlabeled peptide. These results are also included in Figure 4. After the peptide had incorporated, the monolayers were collected directly into scintillation vials and counted. Control experiments showed that when the glass capillary was presaturated with BSA, no radioactivity was lost during transfer. Aliquots of the subphase were also counted. The results of these experiments are summarized in Table I. Although there was considerable scatter in the data, an average area per molecule p25 of 560 ± 170 Å^2 could be determined. No significant dependence on peptide concentration or lipid composition was detected.

Pressure–area isotherms of pure POPG monolayers and mixed POPG/p25 monolayers are shown in Figure 5. p25 was incorporated at 0.25 μM and 25 mN/m. After incorporation was complete, the monolayer was expanded and recompressed at a rate of 5 cm^2/min. Below 25 mN/m some hysteresis was observed at this rate, yielding slightly larger areas during compression than during decompression. However, exactly the same curves were reproduced in a second decompression/compression cycle. Only when the monolayer was compressed to pressures well above 35 mN/m, were considerable amounts of peptide lost to the subphase. Some peptide reincorporated upon decompression at about 23 mN/m. The pressure–area isotherm of pure POPG is typical for lipid monolayers in the "fluid" or "liquid-expanded" state (Phillips & Chapman, 1968; Demel et al., 1972) and is virtually identical with the pressure–area curve of POPC (not shown). A calculated difference curve, POPG/p25 minus POPG, shows that there is little compressibility and/or incorporation of p25 under these nonequilibrium conditions.

Discussion

The molecular mechanism of protein import into mitochondria is largely unknown. However, some key components have been identified. It is well established that in most cases N-terminally linked signal peptides are required to target proteins to the correct location in mitochondria, namely, the matrix, the inner or outer mitochondrial membrane, or the intramembrane space. The minimal requirements for the primary structure of signal peptides are currently investigated by a genetic approach using "fusion proteins", i.e., constructs between various presequences and a protein that is normally not imported [see, e.g., Hurt & van Loon (1986)]. Still less is known about the determinants located on the membrane. In this paper I show that a synthetic signal peptide and synthetic lipids alone are sufficient for binding and incorporation of the peptide to a model membrane. Membrane binding and incorporation naturally are the fist steps in any protein translocation mechanism. The peptide used in this study, "p25", exactly corresponds to the signal sequence of the precursor of subunit IV of yeast cytochrome *c* oxidase. A mon-

olayer technique, which measures the area increase at constant surface pressure, was used to measure peptide incorporation. This method is very sensitive and allows one to detect some tens of picomoles of peptide incorporated into the monolayer. It is a direct thermodynamic method and the signal is easily interpreted and quantified—in contrast to many spectroscopic techniques. The area increase in a monolayer at constant surface pressure directly reflects the amount of solute partitioning from the aqueous subphase into the monolayer [if the average surface area occupied by the peptide in the monolayer is (surface) concentration independent, which is believed to be the case; see Table I and below]. Solutes like metal ions or polylysine, which only adsorb to the head group region of the phospholipid monolayers, do not cause significant changes in monolayer area or pressure (provided they do not induce lipid-phase transitions) (Demel et al., 1973).

Lipid-specific interactions and true incorporation into the lipid phase should be measured at monolayer pressures higher than the equilibrium surface pressure of the peptide itself. p25—as many other proteins—is surface-active. When injected into phosphate-buffered saline under a clean air–water interface, it builds up a surface pressure of 18 mN/m. This is less than 24.5 mN/m for melittin (a membrane-active 26-residue peptide of bee venom; Sessa et al., 1969) but more than 12–14 mN/m for some cardiotoxins (Bougis et al., 1981) or 6 mN/m for glycophorin (van Zoelen et al., 1977). The relatively high surface activity of p25 is quite plausible in view of its proposed "amphiphilic" α-helical structure. Therefore, all phospholipid monolayer incorporation experiments were carried out at surface pressures greater than 20 mN/m.

Taken together, Figures 2–4 demonstrate conclusively that p25 specifically interacts with natural and synthetic phospholipid monolayers. The signal peptide does not just adsorb to the membrane surface (head group region) but instead inserts into the hydrophobic region of the monolayer up to high surface pressures (Figure 3). The "limiting pressure" at which no further peptide is incorporated into a POPC–POPG (1:1) monolayer was 36.4 mN/m. In monolayers a bilayer "equivalence pressure" may be defined as that pressure at which the lipid density in the monolayer is identical with the lipid density in bilayers of the same lipid.[2] The bilayer equivalence pressure of phospholipid monolayers is thought to be 30–34 mN/m (Demel et al., 1975) or possibly somewhat lower (24.5 mN/m; Schindler, 1979). From this comparison it is clear that the peptide–lipid interactions observed in these monolayer experiments are strong enough for significant incorporation of p25 into phospholipid bilayers.

Although p25 incorporated into pure POPC monolayers, a strong enhancement of incorporation was observed when negatively charged phospholipids were present in the monolayer (Figures 2 and 4). This effect was more pronounced at 25 mN/m (Figure 4) than at 21 mN/m (Figure 2). These experiments also suggest that some synthetic phospholipids or mixtures of them are just as good substrates for signal peptide incorporation as the natural mitochondrial phospholipid mixture. Mitochondrial lipids mainly consist of phosphatidylcholine (40%), phosphatidylethanolamine (35%), and cardiolipin (18%) (Daum, 1985), with palmitic and oleic acid as the two predominant fatty acyl chains. As far as chain composition and head group charge density is concerned, the synthetic mixture of 80% POPC and 20% POPG should model the natural lipid composition quite well.

For further analysis and interpretation of the monolayer incorporation data, it was important to determine the average molecular area and the orientation of the signal peptide in the monolayer. The average molecular area of p25 in the monolayer was 560 ± 170 Å^2 as determined with ^{14}C-labeled peptide. The cross sectional area of an α-helix is, depending on the amino acid side chains, of the order of 50–200 Å^2. Thus, the projected areas of the α-helices in the membrane-spanning protein bacteriorhodopsin are about 80–100 Å^2 (Hayward & Stroud, 1981). These areas are much smaller than the area obtained in the p25 monolayer experiments. However, an arrangement of the putative 25-residue helix *parallel* to the membrane surface is consistent with the experimental results. With a pitch of 1.5 Å per residue and an average helix diameter of 12 Å, the α-helix projects sideways onto a surface of 450 Å^2—well within the limits of the observed value. A parallel arrangement is also favored by the amphiphilic nature of the p25 helix, allowing for hydrophobic contacts with the fatty acyl chains (and possibly air) and hydrophilic and electrostatic interactions with the phospholipid head groups and water. The molecular areas of POPC and POPG at 25 mN/m and 21 °C were both 70 Å^2 on 0.1 M NaCl and 10 mM NaP_i, pH 7.0, as determined from their pressure–area isotherms (Figure 5). No monolayer data are available for these lipids in the literature, but literature values for DLPG (66 Å^2; Tocanne et al., 1974) and DMPG (67 Å^2; Mombers et al., 1980) under similar conditions, and the fact that the introduction of one cis double bond causes an area increase of 3–4 Å^2 (Philips & Chapman, 1968; Demel et al., 1972), are in excellent agreement. Furthermore, DMPC and DMPG isotherms on the same buffer subphase were virtually indistinguishable from each other (Mombers et al., 1980). As an aside, it is worth mentioning that the value of 70 Å^2 also corresponds very closely to the molecular area of mono- and diunsaturated lecithins in bilayers (68–73 Å^2) as determined by X-ray diffraction (Lewis & Engelman, 1983; White & King, 1985).

Knowing the molecular area of the peptide and the lipids in the monolayer and assuming additive molecular areas of the components, the plots in Figure 4 are transformed into calibrated incorporation isotherms: moles of incorporated peptide/moles of phospholipid (r) are plotted vs. the free peptide concentration in the subsolution (c_p^f). These isotherms will now be analyzed in terms of a simple combined incorporation/binding model of the general form

$$P \underset{}{\overset{\gamma_1}{\rightleftharpoons}} P_m \overset{K_2,\ +nL^-}{\rightleftharpoons} PL_n^-$$

In this model, the peptide partitions from the aqueous phase into the monolayer (reaction 1) and the partitioned peptide specifically binds n negatively charged lipids in the monolayer (reaction 2). The partition equilibrium is

$$\gamma_1 = (c_p^m)'/c_p^f = (c_p^m/c_p^f)(V_{H_2O}/V_L) \qquad (1)$$

with c_p^f and c_p^m being the free and incorporated peptide concentrations relative to water, V_{H_2O} and V_L the volumes of the aqueous and lipid phase, and $(c_p^m)'$ the incorporated peptide concentration relative to lipid volume, respectively. It seems to be justified to neglect a possible activity coefficient at the low peptide concentrations employed here. The equilibrium constant for partitioning is defined as

$$K_1 = \gamma_1 \bar{V}_L = r_1/c_p^f[M^{-1}] \qquad (2)$$

where $r_1 = c_p^m/c_L^0$, $\bar{V}_L = (V_L/V_{H_2O})/c_L^0$ is the partial molar volume, and c_L^0 is the total lipid concentration. If the binding

[2] The "equivalence pressure" is not necessarily identical with but is probably close to the thermodynamically defined "equilibrium pressure", where the free energy of a lipid in the monolayer equals the lipid free energy in the bilayer.

sites are independent and identical, then the binding constant is

$$K_2 = n[PL_n^-]/[P_m][L^-] = nc_p^b/c_p^m c_L^{-f} \quad (3)$$

The amount of peptide bound to negatively charged phospholipid (mole fraction x) is

$$r_2 = c_p^b/c_L^0 = xc_p^b/c_L^{-0} \quad (4)$$

Mass conservation requires

$$c_L^{-0} = c_L^{-f} + nc_p^b \quad (5)$$

and with $m = n/x$ the binding equation becomes

$$K_2 = mr_2/c_p^m(1 - mr_2) = mr_2/r_1c_L^0(1 - mr_2) \quad (6)$$

The observed signal (total peptide incorporated) is $r = r_1 + r_2 = K_1c_p^f + r_2$, and with this the following incorporation/binding equation is obtained:

$$K_2 = m(r - K_1c_p^f)/K_1c_L^0c_p^f[1 - m(r - K_1c_p^f)] \quad (7)$$

Equation 7 may be put in a linear form:

$$(r - K_1c_p^f)/K_1c_L^0c_p^f = K_2[1/m - (r - K_1c_p^f)] \quad (8)$$

If $(r - K_1c_p^f)/K_1c_L^0c_p^f$ is plotted vs. $r - K_1c_p^f$, a straight line should be obtained with a slope of $-K_2$ and an x-axis intercept of $1/m$.

For data analysis, we first note that incorporation in all three isotherms begins only above a critical peptide concentration $c_p^* = 3.75 \times 10^{-8}$ M. The reason for c_p^* being nonzero is not clear, but the most simple and most likely explanation is a constant background of nonspecific binding of p25 to the Teflon walls. Cooperative aggregation of peptide monomers in the plane of the membrane above a certain critical concentration could produce a similar effect (Schwarz et al., 1986). Thus, the free peptide concentration is taken as $c_p^f = c_p^0 - rc_L^0 - c_p^*$, and r is computed from the raw data with $r = (a_L/a_p)(\Delta A/A_i) = 0.125\Delta A/A_i$. The total lipid concentration is $c_L^0 = A_i/(a_L N_A V_{H_2O}) = 3.80 \times 10^{-7}$ M (a_L and a_p = molecular areas of lipid and peptide, respectively; A_i = initial monolayer area; ΔA = monolayer area increase; N_A = Avogadro's number). K_1 was determined by fitting the pure POPC data to the partition equation (2) and was $\sim 2.7 \times 10^4$ M^{-1}. With this value for K_1 the POPG and POPC–POPG (80:20) data were fit to eq 8, yielding binding constants $K_2 = 5.5 \times 10^8$ and 18×10^8 M^{-1} and binding stoichiometries $n = 8$ and 6, respectively. The values for pure POPG are probably more reliable than those given for 20% POPG, because there is less scatter in the POPG data and better fits were obtained. Binding stoichiometries of 6–8 are sufficient to compensate for all charge on the peptide (+6). About 10 lipid molecules (70 Å^2) are needed to fully solvate one peptide molecule with molecular dimensions of 12×37.5 Å^2 (cf. above). This is only a little more than the measured stoichiometry at maximum binding. Some interactions between peptides may occur in the monolayer and could account for the observed difference. Because of their identical chain composition it is reasonable to use the same K_1 for POPC and POPG. K_1 may be converted back into an ordinary partition coefficient $\gamma_1 = K_1/\bar{V}_L \approx 3.2 \times 10^4$, where $\bar{V}_L = N_A a_L d \approx 0.84$ dm^3/mol is the partial molar volume of POPC in the monolayer (with the molecular area of POPC at 25 mN/m, $a_L = 70$ Å^2, and the POPC monolayer thickness $d = 20$ Å). The binding constant K_2 of $5–18 \times 10^8$ M^{-1} for six electrostatic bonds yields an average binding energy of 8–9 kJ/mol per bond formed. This agrees with typical energies for ion-pair formation in proteins or the binding of Mg^{2+} to ATP^{4-} [cf. Cantor & Schimmel (1980)].

Obviously, more complicated binding and incorporation models could be employed to describe the insertion of peptides into model membranes. Statistical effects for large ligand adsorption (Stankowski, 1983a,b, 1984) may also become important for membrane incorporation. However, fits to analogues of eq 7 and 8, using $K_2 = [PL_n]/([P_m][L]^n)$ [cf. Stankowski (1983a)] instead of eq 3, were, for any $n > 1$, worse than the ones obtained here. The effects of the monolayer surface potential on signal peptide incorporation are not clear at present. A rigorous application of the Gouy–Chapman theory [see, e.g., McLaughlin (1977)] should be questioned in view of the large size of the peptide (which certainly does not represent a point charge of +6) and the short Debye length (~ 10 Å) in 0.1 M NaCl. If nevertheless surface potentials contribute to the incorporation of signal peptide, the given binding constants are upper limits.

At least three things happen when the signal peptide interacts with a membrane (the temporal sequence of these events is presently not known): (1) The peptide changes conformation from random coil to an α-helix. (2) The peptide inserts into monolayers with its helical axis parallel to the membrane surface, at a depth to allow for the formation of hydrophobic and electrostatic contacts. (3) Complexes between negatively charged phospholipids and the positively charged peptide residues are formed, and as a consequence, the incorporation equilibrium is shifted toward the membrane side. Of course, some of these observations have been made with phospholipid monolayers, which at 25–30 mN/m are a model for only one-half of a lipid bilayer. Nevertheless, the initial events of the interaction of signal peptides with membranes may be modeled correctly. Future binding and incorporation experiments with phospholipid bilayers are necessary to establish the correct relationship to the monolayer studies presented here.

The ability of negatively charged membrane–water interfaces to bind and incorporate polypeptides upon a conformational change from random coil to α-helix is not unique to the mitochondrial signal peptide studied here. It has also been observed with a 25-residue signal sequence from an *E. coli* outer membrane protein (Briggs & Gierasch, 1984; Briggs et al., 1985), the 23-residue leader peptide of the M13 coat protein (Shinnar & Kaiser, 1984), and with apocytochrome *c* (Rietveld et al., 1985). Interestingly, apocytochrome *c* is synthesized in the cytoplasm directly in its mature length. During or immediately after its translocation across the outer mitochondrial membrane the heme group is covalently attached to form the highly structured holocytochrome *c*. All three, the *E. coli* Lam B, the M13 signal sequences, and apocytochrome *c*, bear an excess of positive charges. Charged amphiphilic helices are in fact quite abundant secondary structures in membrane-active polypeptides (Eisenberg, 1984; Kaiser & Kézdy, 1984). Some prominent examples include melittin and many other peptide toxins and hormones. Some of them have been shown to bind to negatively charged phospholipid membranes with high affinity, but in most cases the association constants were too high ($>10^6$–10^7 M^{-1}) to be determined from measurements of intrinsic fluorescence quenching. Even in many large integral membrane proteins that span the membrane several times (like bacteriorhodopsin or lactose permease from *E. coli*), amphiphilic helices are quite frequent. Whether they incorporate by a mechanism similar to that of the mitochondrial signal peptides is an interesting question for the future.

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