Membrane insertion and lateral diffusion of fluorescence-labelled cytochrome *c* oxidase subunit IV signal peptide in charged and uncharged phospholipid bilayers

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The synthetic 25-residue signal peptide of cytochrome *c* oxidase subunit IV was labelled with the fluorophor 7-nitrobenz-2-oxa-1,3-diazole (NBD) at its single cysteine residue. Addition of small unilamellar vesicles of 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) to the labelled peptide resulted in a shift of the NBD excitation and emission spectra to shorter wavelengths. Binding of the peptide to the vesicles was measured by the increase in the fluorescence emission yield. A surface partition constant of $(3.9 \pm 0.5) \times 10^3 \text{ M}^{-1}$ was derived from these titrations. When the membrane contained, in addition to POPC, negatively charged 1-palmitoyl 2-oleoyl phosphatidylglycerol (POPG), the NBD fluorescence spectra were further shifted to shorter wavelengths and exhibited increased quantum yields. The apparent partition constants were increased to 10^4 – 10^5 M^{-1} for vesicles with 20 or 100 mol % POPG. Lateral diffusion of the peptide was measured by fluorescence recovery after photobleaching in multibilayers of POPC, POPG, POPC/POPG (4:1) and 1,2-dimyristoyl phosphatidylcholine. The lateral diffusion coefficients of the peptide in bilayers of POPC ($8 \times 10^{-8} \text{ cm}^2/\text{s}$ at 21 °C) were 1.5–1.6-fold greater than those of NBD-labelled phospholipids ($5 \times 10^{-8} \text{ cm}^2/\text{s}$ at 21 °C), but 1.5–1.8-fold smaller ($3 \times 10^{-8} \text{ cm}^2/\text{s}$ in 20 % POPG and at 21 °C) than the lipid diffusion coefficients in the negatively charged bilayers. It is concluded that the signal peptide associates with phospholipid bilayers in two different forms, which depend on the lipid charge. The experiments with POPC bilayers are well explained by a model in which the peptide partitions into the region of the phospholipid head-groups and diffuses along the membrane/water interface. If POPG is present in the membrane, electrostatic attractions between the basic residues of the peptide and the acidic lipid head-groups result in a deeper penetration of the bilayer. For this case, two models that are both consistent with the experimental data are discussed, in which the peptide either forms an oligomer of three to six partially helical membrane-spanning monomers, or inserts into the bilayer with its amphiphilic helical segment aligned parallel to the plane of the membrane and located near the head-group and outer hydrocarbon region of the bilayer.

INTRODUCTION

Signal sequences are transient extensions of the *N*-termini of many integral membrane proteins and proteins that are transported across one or more membranes before they reach their final destination. After proper membrane assembly or passage through the membrane, most signal sequences are proteolytically removed from the mature protein by specific signal peptidases. The signal sequences appear to fulfil two separate functions: they are recognized by an appropriate receptor system on their respective target membrane(s), and they can interact with several membrane components in order to facilitate the assembly and translocation of the mature protein in the lipid-bilayer membrane. Quite a lot of work has concentrated on the targeting function of signal sequences from different sources, and several excellent reviews concerned with this topic have appeared [1–4]. It is clear that the primary structures of the signal sequences of different proteins destined for the same membrane or cellular compartment are not highly conserved. Only general features such as charge and polarity distributions of the residues are common in a given class of signal sequences. For instance, the *N*-termini of the protein precursors targeted to the matrix of mitochondria are characterized by many positively charged and apolar residues, which are arranged in a sequence

that allows for the formation of amphiphilic α -helices. Signal-sequence receptors are known to exist in the endoplasmic-reticulum membrane [5] and were reported to occur in chloroplast-envelope contact zones [6]. A 42 kDa protein of the mitochondrial outer membrane that cross-links to an engineered precursor protein on its way to the mitochondrial matrix [7] and a 19 kDa polypeptide with receptor function [7a] have also been identified recently on the mitochondrial surface. The low degree of conservation within the different classes of signal sequences requires a large range of substrate-specificities for these proteins.

Parallel to these observations, it has also been found that isolated synthetic signal peptides of bacterial origin can readily associate with artificial bilayers consisting solely of phospholipids (see ref. [8] for a review). The major conclusions of these studies were that the signal peptides are incorporated into lipid monolayers up to high limiting pressures and that they change their secondary structure (mostly into α -helices) when they associate with lipid surfaces. Similar results were obtained with mitochondrial signal sequences. Synthetic presequences of cytochrome *c* oxidase subunit IV of different lengths were found to insert into phospholipid monolayers at surface pressures above 30 mN/m, to become about 40 % α -helical in micelles of SDS and to disrupt phospholipid vesicles at peptide concentrations greater than about $1 \mu\text{M}$ [9]. The concentration at

Abbreviations used: coxIV-25, 25-residue signal peptide of yeast cytochrome *c* oxidase subunit IV; f.r.a.p., fluorescence recovery after pattern photobleaching; IANBD, *N*-[2-(iodoacetoxymethyl)amino-7-nitrobenz-2-oxa-1,3-diazole]; NBD, 7-nitrobenz-2-oxa-1,3-diazole; NBD-coxIV-25, NBD-labelled coxIV-25; NBD-eggPE, egg *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; DMPC, 1,2-dimyristoyl phosphatidylcholine [1,2-dimyristoyl-*sn*-glycero(3)phosphocholine]; POPC, 1-palmitoyl 2-oleoyl phosphatidylcholine [1-palmitoyl-2-oleoyl-*sn*-glycero(3)phosphocholine]; POPG, 1-palmitoyl 2-oleoyl phosphatidylglycerol [1-palmitoyl-2-oleoyl-*sn*-glycero(3)phosphoglycerol].

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which half-maximal vesicle disruption occurred depended on the trans-bilayer potential, and the peptide was most effective when this potential was negative inside the vesicles (the mitochondrial membrane potential is also negative inside). Like the bee venom melittin, the 25-residue presequence peptide exhibited haemolytic activity on erythrocytes in a potential-sensitive manner and caused the aggregation of band 3 protein in the erythrocyte membrane [10]. The 27-residue signal peptide of ornithine carbamoyltransferase has also been found to insert into synthetic phospholipid vesicles, but, surprisingly, this association did not depend on the membrane potential [11]. It was further concluded from n.m.r. experiments that the *N*-terminal half of the 25-residue cytochrome *c* oxidase peptide (coxIV-25) took a helical structure whereas the *C*-terminal half did not take a regular secondary structure in dodecylphosphocholine micelles [12]. Experiments with the use of a monolayer technique at constant surface pressure revealed that the incorporation of coxIV-25 into phospholipid monolayers strongly depended on the presence of negatively charged lipids in the monolayer [13]. By determining the area per peptide in the monolayer, it was found that the peptide was incorporated into the monolayers with its α -helical segment oriented parallel to the plane of the membrane. In order to test whether these observations can be generalized to lipid bilayers, we have now labelled coxIV-25 with the fluorophore NBD at its single cysteine residue and measured its incorporation into charged and uncharged lipid vesicles. In addition, the lateral diffusion coefficients of the peptide in different lipid environments have been determined. With these experiments we are able to distinguish between two different membrane-bound forms of NBD-coxIV-25.

EXPERIMENTAL

Materials

POPC, POPG and NBD-eggPE were from Avanti Polar Lipids (Birmingham, AL, U.S.A.) and some POPC was from Berchtold (Switzerland). All lipids were shown to be pure by silica-gel t.l.c. and used without further purification. IANBD was purchased from Molecular Probes (Eugene, OR, U.S.A.). The sequence of the mitochondrial signal peptide coxIV-25 is shown in Fig. 1. This peptide was synthesized as described previously [9] and was kindly donated by Dr. G. Schatz.

Peptide labelling and purification

A 100 nmol portion of purified synthetic coxIV-25 was dissolved in 0.1 M-ammonium acetate buffer, pH 8.1, or in water to give a 0.5 mg/ml solution. In order to reduce potentially oxidized cysteine residues, this solution was incubated with 25 nmol of dithiothreitol at 37 °C under Ar for 1 h. Subsequently a 3-fold excess of IANBD (450 nmol) over the total of thiol groups was added and the coupling reaction was allowed to proceed for 1 h under Ar at 37 °C.

Unlabelled and labelled peptides were purified on a C_4 reverse-phase h.p.l.c. column (bead size 7 μ m, pore size 50 nm) (Machery-Nagel, Oensingen, Switzerland). A 1% (v/v)/min gradient of acetonitrile versus 0.05% (v/v) trifluoroacetic acid in water was used. Peptide concentrations were determined by quantitative amino acid analysis. NBD concentrations were measured spectrophotometrically at 494 nm. The absorption coefficient of IANBD in water was determined to be 27600 M⁻¹·cm⁻¹ and was assumed not to change upon coupling to the peptide. The degree of labelling, defined as the concentration of the label divided by the peptide concentration after separation from all free label, was 97 $\pm$ 8%.

Incorporation isotherms

The incorporation of NBD-coxIV-25 into vesicles was measured fluorimetrically on an SLM 8000 spectrofluorimeter. Small unilamellar vesicles were prepared as follows. A 5 μ mol or 10 μ mol portion of the desired lipids was mixed in chloroform. The solvent was evaporated with a flow of N₂ and the lipids were dried in a high vacuum. The lipids were resuspended in 1 ml of 10 mM-Pipes/NaOH buffer, pH 7.3, containing 0.15 M-NaCl and 0.002% NaN₃ and sonicated on a tip sonicator for 20–30 min at 10 °C under a flow of N₂. A 2.5 ml portion of a 0.27 μ M solution of NBD-coxIV-25 in 10 mM-Pipes/NaOH buffer, pH 7.3, containing 0.15 M-NaCl was left to equilibrate at 20 °C in a stirred 1 cm fluorescence cuvette for at least 20 min. The fluorescence intensity was measured with the use of excitation at 490 nm and emission at 520 nm with 8 nm slits in the excitation and emission pathways. Appropriate portions of the concentrated vesicle suspensions were added to this solution, the sample was allowed to equilibrate for 20–30 min, and the intensities were measured with the same spectrometer settings. The resulting incorporation isotherms were fitted to the different binding models by Monte Carlo and non-linear least-squares Marquardt procedures provided in the NLFit software (E.T.H., Zurich, Switzerland) on a Macintosh II computer.

Lateral diffusion measurements

The lateral diffusion experiments were performed on stacks of fully hydrated bilayers (multibilayers). Multibilayers were prepared by the following method. The desired lipids (1.25 μ mol) were mixed in chloroform. At this stage, NBD-eggPE was added at a molar ratio of 1:1250 to the samples that were to be used for lipid diffusion measurements. The solvent was evaporated with a flow of N₂ and the lipids were dried in a high vacuum. The lipids were resuspended carefully in 25 μ l of water. For measurements of peptide diffusion, the water contained 0.68 nmol of NBD-coxIV-25, which resulted in a peptide/lipid ratio of 1:1800. This suspension was divided into two equal parts and put on two coverslips. The samples were then dried in a closed argon chamber containing a saturated LiCl solution at room tem-

Fig. 1. Amino acid sequence of coxIV-25

All charges are indicated. The fluorescent label NBD is attached to the cysteine residue at position 19.

Fig. 2. Purification of NBD-coxIV-25 by h.p.l.c.

(a) Elution profile of the starting material. CoxIV-25 was purified before the labelling reaction. (b) Elution profile of NBD-coxIV-25 after labelling and purification. The samples were run on a reverse-phase C_4 column with a 1% (v/v) min gradient of acetonitrile versus 0.05% (v/v) trifluoroacetic acid in water. The retention time of the peptide remains nearly unchanged after NBD coupling.

perature (42 °C for DMPC) for 3–4 h. They were mounted, lipids down, on a microscope slide with double-stick tape. The lipids were rehydrated in the same argon chamber containing a 0.1 M-NaCl solution instead of LiCl solution for about 60 h at 42 °C. A drop of 1 M-NaCl was put between the microscope slide and the coverslip. Care was taken to avoid contact of this drop with the multibilayers. The samples were sealed with vacuum grease to give a closed chamber of constant and high relative humidity (97%) and were left at room temperature for a final equilibration of at least 15 h.

The lateral diffusion coefficients and mobile fractions were measured by f.r.a.p. [14] as described in Tamm [15]. Typically, 30 observation pulses followed a single bleach pulse. As a consequence of the construction of the sample cell used in this work, the accuracy of the temperature control of the bilayers is estimated to be ± 2 °C. For each lipid composition, for each sample and at each temperature, three to 15 individual experiments were averaged and yielded standard deviations of less than 10% in most cases.

RESULTS

Fluorescent probe labelling

The thiol-reactive fluorophore IANBD was coupled to the single cysteine residue at position 19 of the synthetic coxIV-25. Both the unlabelled peptide and the labelled peptide (NBD-coxIV-25) were purified by reverse-phase h.p.l.c., and two representative elution profiles are shown in Fig. 2. The purified reaction product was eluted as a single peak with a retention time that was very similar to that of the main peak of the crude unlabelled material. This suggests that the label does not greatly change the overall polarity of the peptide. Since IANBD does not label free amino groups at pH 8 or below, NBD-coxIV-25 is expected to retain the six positive charges at physiological pH. The incorporation of NBD-coxIV-25 into charged phospholipid monolayers (results not shown) was similar to previous measurements of the incorporation of coxIV-25 into monolayers [13]. This result further supports the notion that the NBD label does not drastically alter the biophysical behaviour of this peptide.

Fig. 3. Fluorescence excitation and emission spectra of NBD-coxIV-25 in buffer and associated with vesicles of different lipid composition

The figure shows fluorescence spectra of NBD-coxIV-25 in 10 mM-Pipes buffer, pH 7.3, containing 0.15 M-NaCl and 0.002% NaN_3 (····; $\lambda_{\text{exc. (max.)}} = 495$ nm, $\lambda_{\text{em. (max.)}} = 532$ nm), with 0.66 mM-POPC small unilamellar vesicles added (—; $\lambda_{\text{exc. (max.)}} = 488$ nm, $\lambda_{\text{em. (max.)}} = 523$ nm; 5× reduced ordinate), or with 0.66 mM-POPC/POPG (4:1) small unilamellar vesicles added (—; $\lambda_{\text{exc. (max.)}} = 485$ nm, $\lambda_{\text{em. (max.)}} = 521$ nm; 10× reduced ordinate). The spectra of IANBD in Pipes buffer are shown for reference (····; $\lambda_{\text{exc. (max.)}} = 495$ nm, $\lambda_{\text{em. (max.)}} = 532$ nm). Excitation spectra are recorded at an emission wavelength of 520 nm and emission spectra at an excitation wavelength of 490 nm.

Fluorescence spectra of NBD-coxIV-25

Fluorescence excitation and emission spectra of NBD-coxIV-25 in different environments are presented in Fig. 3. In aqueous buffer NBD-coxIV-25 exhibits excitation and emission maxima at 495 ± 1 nm and 531 ± 1 nm (16 experiments) respectively (····

Fig. 4. Isotherms of NBD-coxIV-25 incorporation into small unilamellar vesicles of different lipid composition

Vesicles of POPC (●), POPC/POPG (4:1) (■) and POPG (◆) were titrated to $0.27 \mu\text{M}$ -NBD-coxIV-25 in Pipes buffer, pH 7.3, containing 0.15 M NaCl and 0.002 % NaN_3 . Each curve represents an individual lipid titration experiment. The measuring temperature was 20°C .

lines). The same values were measured for IANBD in water (.....). When small unilamellar phospholipid vesicles of POPC were added, both maxima were shifted towards shorter wavelengths ($487 \pm 1 \text{ nm}$ and $524 \pm 1 \text{ nm}$; means $\pm$ s.d. for five experiments) and the relative fluorescence intensities were increased (— lines; note the different scales of the ordinate in Fig. 3). When the vesicles also contained the negatively charged phospholipid POPG (20 %), the excitation and emission maxima were further decreased to $484 \pm 1 \text{ nm}$ and $521 \pm 1 \text{ nm}$ (means $\pm$ s.d. for four experiments) respectively, and the fluorescence intensity was further enhanced (— lines). The observed wavelengths of the spectral maxima in the presence of the POPC/POPG (4:1) vesicles were similar to those measured with NBD-coxIV-25 dissolved in propan-2-ol, but they were still greater than those measured in chloroform (results not shown). These observations demonstrate that the peptide associates with pure POPC and negatively charged POPC/POPG (4:1) vesicles with the label being in an environment of intermediate polarity.

Binding of NBD-coxIV-25 to lipid vesicles

The effect of the increased fluorescence quantum yields of NBD in membranes was then exploited to measure incorporation isotherms of the peptide into vesicles of different phospholipid composition. Small unilamellar vesicles of POPC, POPC/POPG (4:1) and POPG were titrated to $0.27 \mu\text{M}$ -NBD-coxIV-25 (Fig. 4). In the titrations with POPC, a relatively small and steady increase of the fluorescence intensities was observed over the whole measured concentration range, i.e. up to a lipid/peptide ratio of 6000:1. When the peptide was titrated with negatively charged vesicles, the fluorescence intensities increased to much higher values within a narrow concentration range and reached a plateau value at lipid/peptide ratios of about 1000:1. These observations indicate that the positively charged peptide was strongly attracted by the negatively charged lipids. A large part of this electrostatic attraction was already achieved with vesicles containing 20 % POPG.

Lateral diffusion of NBD-coxIV-25 in lipid bilayers

In order to learn more about the state of association of NBD-coxIV-25 with lipid model membranes, its lateral diffusion coefficient and mobile fraction were measured by f.r.a.p. in stacks of hydrated bilayers of various lipid compositions. These

results were compared with f.r.a.p. measurements on the fluorescent phospholipid analogue NBD-eggPE in multibilayers of the same lipid compositions. Fig. 5 shows three representative fluorescence recovery curves. Two are the fluorescence recoveries obtained from NBD-coxIV-25 in POPC and POPC/POPG (4:1) bilayers, and one is the recovery curve of NBD-eggPE in POPC. All three f.r.a.p. curves could be fitted by single exponentials (continuous lines), indicating a single diffusing species. The lateral diffusion coefficient, determined from the exponential time constant of the best-fit curves [14], was $5.2 \times 10^{-8} \text{ cm}^2/\text{s}$ for NBD-eggPE in POPC bilayers at 21°C . The same lateral diffusion coefficient was obtained with NBD-eggPE in membranes containing 20 % POPG (see Table 1). In contrast with lipid diffusion, the lateral diffusion coefficients of the peptide depended on the amount of POPG present in the bilayers. With pure POPC bilayers, the diffusion coefficient of the peptide ($8.1 \times 10^{-8} \text{ cm}^2/\text{s}$) was greater than the lipid diffusion coefficient, whereas this value was lower than lipid diffusion in bilayers containing 20 % POPG ($2.8 \times 10^{-8} \text{ cm}^2/\text{s}$).

The lateral diffusion parameters of the labelled lipid and peptide were measured at different temperatures and in membranes of different lipid compositions. These data are summarized in Table 1. Lipid diffusion in multibilayers of POPC, POPC/POPG (4:1), POPG and DMPC (30°C) was fast (10^{-8} – $10^{-7} \text{ cm}^2/\text{s}$) with mobile fractions greater than 90 %, which is typical for bilayers in a fluid or liquid-crystalline state [16]. At 15°C the DMPC bilayers were in a solid (gel) state with 50 % of the lipid probe diffusing with a lateral diffusion coefficient of $1.7 \times 10^{-10} \text{ cm}^2/\text{s}$, which is also in agreement with published results [16]. Addition of 20 % POPG to the POPC bilayers did not change the lateral diffusion coefficients or mobile fractions of NBD-eggPE significantly. However, the lateral diffusion coefficient of this probe was increased in bilayers of pure POPG compared with POPC by about 20–25 %. This effect may be due to the high electrostatic repulsion between the lipids in the POPG bilayers.

The lateral diffusion coefficients of NBD-coxIV-25 were 1.5-fold greater than the lipid diffusion coefficients in pure POPC multibilayers at all temperatures and also in DMPC multibilayers at 30°C . However, in the presence of negatively charged lipids, the diffusion coefficient of the peptide was decreased 1.5–1.8-fold compared with the lipid diffusion coefficient. A remarkable result

Fig. 5. Lateral diffusion of NBD-coxIV-25 and of NBD-eggPE in multibilayers of different lipid composition

Fluorescence recovery curves are shown for NBD-coxIV-25 in POPC (●) and POPC/POPG (4:1) (■) and for NBD-eggPE in POPC (○). The time scale at the top of the Figure refers to the time that would apply if all fluorescence recoveries had been measured with a pattern of $12.7 \mu\text{m}$ periodicity on the membrane. The lateral diffusion coefficients of the depicted curves as derived from single-exponential fits are $8.2 \times 10^{-8} \text{ cm}^2/\text{s}$ (●), $5.0 \times 10^{-8} \text{ cm}^2/\text{s}$ (○) and $2.7 \times 10^{-8} \text{ cm}^2/\text{s}$ (■). The measuring temperature was 21°C .

Table 1. Lateral diffusion coefficients and mobile fractions of NBD-eggPE and NBD-coxIV-25 in multibilayers of different lipid compositions

D is the lateral diffusion coefficient; n is the number of samples, and m is the number of measurements per sample.

Lipid composition	Temperature ($^\circ\text{C}$)	Lipid diffusion				Peptide diffusion			
		$D (\mu\text{m}^2/\text{s})$	Mobile fraction (%)	n	(m)	$D (\mu\text{m}^2/\text{s})$	Mobile fraction (%)	n	(m)
POPC	15	2.24 ± 0.15	94.8 ± 2.6	6	(5–9)	3.32 ± 0.48	89.8 ± 4.0	5	(6–15)
POPC	21	5.19 ± 0.18	95.0 ± 2.4	19	(4–15)	8.07 ± 0.62	88.6 ± 4.4	7	(5–10)
POPC	30	7.17 ± 0.58	91.8 ± 2.4	7	(5–10)	10.88 ± 0.51	87.2 ± 0.8	4	(6–16)
POPC/POPG (4:1)	15	2.40 ± 0.56	89.8 ± 4.0	5	(6–9)	1.60 ± 0.16	91.6 ± 2.2	3	(7–8)
POPC/POPG (4:1)	21	5.06 ± 0.26	94.6 ± 2.6	8	(5–15)	2.79 ± 0.35	89.8 ± 6.1	8	(5–14)
POPC/POPG (4:1)	30	8.30 ± 0.60	93.2 ± 3.8	3	(5–8)	4.79 ± 0.87	86.4 ± 3.6	4	(6–8)
POPG	21	6.34 ± 0.64	93.6 ± 4.2	6	(10)	2.92 ± 0.40	87.0 ± 4.8	6	(8–11)
DMPC	15	0.0173 ± 0.0024	50.2 ± 5.4	4	(6–7)	0.242 ± 0.086	55.0 ± 10.8	7	(3–8)
DMPC	30	6.23 ± 0.68	88.0 ± 3.6	9	(6–9)	12.22 ± 1.05	90.2 ± 3.2	7	(5–8)

was obtained with bilayers of DMPC at 15°C , where a lateral diffusion coefficient of $2.4 \times 10^{-9} \text{ cm}^2/\text{s}$ with a mobile fraction of 55% was measured for NBD-coxIV-25. This value is intermediate between the solid and fluid lipid diffusion coefficients, i.e. it is 14-fold larger and 9–10-fold smaller than the lipid lateral diffusion coefficient in solid (DMPC) and fluid (POPC) membranes at 15°C respectively.

DISCUSSION

The association of amphiphilic peptides with lipid bilayers is most simply described by the partition equilibrium:

$$x_p^m = K_p \cdot c_p^f \quad (1)$$

where $x_p^m = c_p^m/c_L$ is the mole fraction of peptide inserted into the membrane, c_p^f and c_p^m are the free and inserted peptide concentrations respectively, c_L is the concentration of the lipids

of the outer leaflet of the vesicles, and K_p is the partition constant in units of $1/\text{mol}$. c_L is taken as 60% of the total lipid concentration, because about 60% of all lipids are present in the outer leaflet of small unilamellar vesicles and, most likely, only these lipids are accessible to the peptide under conditions of low-to-moderate peptide/lipid ratios and in the absence of a transmembrane potential. This mode of association is also consistent with the results of our lateral diffusion experiments (see below). K_p is related to the dimensionless partition coefficient γ by $K_p = \gamma \cdot \bar{v}_L$, with $\bar{v}_L$ being the partial molar volume of the lipid. The experimental data of Fig. 4 (averaged for each lipid composition) were first fitted to eqn. (1). The best-fit curves are plotted in Fig. 6. This analysis yields apparent partition constants of $(3.9 \pm 0.5) \times 10^3 \text{ M}^{-1}$, $(6.7 \pm 0.4) \times 10^4 \text{ M}^{-1}$ and $(2.6 \pm 0.5) \times 10^5 \text{ M}^{-1}$ for POPC, POPC/POPG (4:1) and POPG respectively. It is evident that excellent fits are obtained with this simple model for the association of NBD-coxIV-25 with POPC and POPC/POPG

Fig. 6. Best fits of the incorporation isotherms with the partition model

The experimental incorporation isotherms of Fig. 4 were averaged for each lipid composition and fitted to eqn. (1). The resulting apparent partition coefficients are $(3.9 \pm 0.5) \times 10^3 \text{ M}^{-1}$ for POPC, $(6.7 \pm 0.4) \times 10^4 \text{ M}^{-1}$ for POPC/POPG (4:1) and $(2.6 \pm 0.5) \times 10^5 \text{ M}^{-1}$ for POPG. The best-fit curves with the Gouy–Chapman partition model are nearly indistinguishable from the ones shown here although the numerical values are quite different (see the text). Monte Carlo and non-linear least-squares Marquardt fits gave identical results.

(4:1) vesicles, whereas the experimental isotherms with POPG vesicles exhibit some systematic deviations from the theoretical curve. Note also that, owing to light-scattering at very high lipid concentrations, the incorporation of the peptide into POPC vesicles could not be measured up to saturation. Therefore the reported partition constant for POPC represents an upper limit. The extrapolated saturation values were 2.9 ± 0.1 , 5.2 ± 0.05 and 5.9 ± 0.05 respectively in our arbitrary fluorescence units, i.e. they were distinctly different for the three sets of data. The 27-residue signal peptide of another mitochondrial protein, ornithine carbamoyltransferase, was reported to bind to phospholipid vesicles that contained various mole fractions of cardiolipin with affinities comparable with the ones determined here [11].

Since the fatty acyl chain compositions of POPC and POPG are identical, the increases of the apparent partition coefficients with increasing amounts of negatively charged lipid must originate from electrostatic interactions of the peptide with these membranes. In order to understand these charge interactions quantitative, the Gouy–Chapman theory of the diffuse ionic double layer near charged lipid/water interfaces (see ref. [17]) has been combined with the partition eqn. (1) [18,19]. By following this Gouy–Chapman partition theory, it is possible to fit the three averaged incorporation isotherms of Fig. 6 with a consistent set of parameters, namely a partition constant of $(3.9 \pm 0.8) \times 10^3 \text{ M}^{-1}$ and an effective charge of the peptide of 1.8 ± 0.1 . These best-fit curves virtually superimpose with those shown in Fig. 6. It has been reported before that the nominal charges of two other polycationic peptides have to be reduced to effective charges in the range 1.8–2.4 in order to describe successfully their binding to charged membranes with the Gouy–Chapman partition model [18,19]. However, the physical reason for this charge reduction is still poorly understood. It might be due to the relatively large size of the peptide compared with the characteristic distance of interaction in the Gouy–Chapman theory (the Debye length at 0.15 M-NaCl is 0.8 nm) and the dispersion of the charges over several residues on the peptide, or alternatively the surface potential may be lower than expected from the theory when multivalent ions instead of univalent ions reside at the membrane/water interface [20]. When our present binding data are fitted with the partition-binding model that has been pre-

viously used to interpret the incorporation of coxIV-25 into charged and uncharged monolayers [13], larger deviations from the experiment are found than with the simple partition and Gouy–Chapman partition models.

Our apparent partition constants of NBD-coxIV-25 in the presence of acidic phospholipids are moderate (10^4 – 10^5 M^{-1}) and about four orders of magnitude lower than reported binding constants of mitochondrial precursors to putative signal sequence receptors (about 10^8 – 10^9 M^{-1} [21,22]). However, if the concentration of ‘high-affinity binding sites’ is about 3–10 pmol/mg of mitochondrial protein [21,22], the concentration of lipids in the cytoplasm-exposed membrane leaflet of mitochondria is 10^4 – 10^5 -fold higher than that of the proteinase-sensitive binding sites, and the membrane lipids may become as efficient as the receptors in binding protein precursors. In fact, considering the low amount of sequence similarity between the different mitochondrial signal sequences, it has been suggested that ‘multiple filtering’ by several signal-sequence-binding components is required to achieve high targeting specificity [3,23,24]. Membrane lipids and signal-sequence-binding proteins in mitochondrial membranes can be such components, which may act in a concerted or sequential mechanism.

The most striking result of our lateral diffusion measurements is the observation of different lateral diffusion coefficients of NBD-coxIV-25 in POPC bilayers with and without the anionic phospholipid POPG. These differences occur at all measured temperatures, and the lateral diffusion coefficients of the peptide are separated from the lipid diffusion coefficients by typically 2–8 standard deviations (Table 1). In pure POPC bilayers and at all temperatures, the lateral diffusion coefficients of NBD-coxIV-25 are larger than those of the lipids themselves, although the molecular mass of NBD-coxIV-25 is more than 3 times larger than that of the lipids. Several mechanisms may be envisaged to explain fast peptide diffusion in POPC multibilayers: (1) the peptide may diffuse in the aqueous layers between the bilayers, (2) lateral transport may occur by a sequence of binding and unbinding reactions, i.e. by hopping back and forth between water and the bilayer {such a mechanism has been demonstrated to be dominant over lateral diffusion for the transport of cytochrome *c* on the surface of mitochondrial membranes at intermediate ionic strengths (50 mM), where 10–20% of all cytochrome *c* is bound to the membrane [25]}, or (3) the peptide may remain associated with the surface of the bilayer and diffuse laterally along this interface. Mechanism (1) can be excluded, because (a) the emission maximum of NBD-coxIV-25 shifts from 532 nm to 523 nm in the presence of POPC, indicating that NBD-coxIV-25 binds to POPC (Fig. 3), and (b) the three-dimensional diffusion coefficient for unhindered diffusion of the peptide in water calculated from the Stokes–Einstein equation is $(2\text{--}3) \times 10^{-6} \text{ cm}^2/\text{s}$ and a limitation of the diffusion space without attractive boundary interactions can only increase the diffusion coefficient. If hopping (mechanism 2) were the main reason for lateral transport, similar apparent lateral diffusion coefficients of the peptide would be expected on the mobile fraction of DMPC below the chain melting-phase transition, as on fluid POPC bilayers. Since at 15 °C the peptide diffused on DMPC bilayers 14 times more slowly than on POPC bilayers (Table 1), and since it can be estimated from the incorporation isotherms of Fig. 4 that more than 99% of all peptide is bound to the membrane under conditions of the lateral diffusion experiments, mechanism (2) can also be excluded. Lateral diffusion along the membrane surface (mechanism 3) is the only remaining explanation of our diffusion results with pure phosphatidylcholine bilayers.

For bilayers containing POPG, more than one interpretation for the decreased lateral diffusion coefficients of NBD-coxIV-25 can be given. Single helices that span the bilayer are known to

diffuse with roughly the same lateral diffusion coefficients as the lipids themselves [26,27]. Also, their cross-sectional areas are very similar to those of single phospholipids in the membrane. Therefore the lateral diffusion coefficients reported here exclude that the peptide diffuses in the bilayer as a single helix oriented perpendicularly to the plane of the membrane. Bacteriorhodopsin with its seven bilayer-spanning helices exhibits in DMPC and at low concentrations lateral diffusion coefficients between $1.4 \times 10^{-8} \text{ cm}^2/\text{s}$ (24.5 °C) and $3.4 \times 10^{-8} \text{ cm}^2/\text{s}$ (32 °C), i.e. it diffuses 2–3 times more slowly than the lipids under identical conditions [28]. Our lateral diffusion coefficients of the peptide in negatively charged membranes are about 1.5–1.8-fold smaller than those of the lipids (Table 1). These results are consistent with a peptide oligomer that diffuses as an entity of about three to six helices each spanning the membrane. Alternatively, the peptide might insert into negatively charged membranes with its α -helical segment oriented parallel to the plane of the membrane. In this case it would penetrate more deeply into the interior of the bilayer than it does in the case of pure POPC bilayers. Coulombic attractions between the negatively charged phosphate groups on the membrane and the positively charged amino groups on the peptide could stabilize this deeper insertion into the bilayer. Although all lateral diffusion measurements were conducted at very dilute peptide concentrations of only 1 peptide molecule per 1800 lipid molecules (i.e. conditions that may favour the monomeric state of the peptide), a final decision as to whether the peptide inserts perpendicularly as an oligomer or horizontally as a monomer [13] into the bilayers containing POPG cannot be made on the basis of the results presented here.

Irrespective of the actual structure of the peptide in the negatively charged membranes, our lateral diffusion experiments prove that NBD-coxIV-25 associates with pure lipid model membranes in at least two different forms, which depend on the charge on the lipids in the membrane. The observed change of the fluorescence quantum yield in charged and uncharged bilayers and the small but reproducible shifts of the excitation and emission wavelengths further support this conclusion. It is noteworthy in this context that a prokaryotic signal sequence (lamB) was found to occur in two different conformations in lipid model membranes [29,30]. However, in that work the two conformations were induced by different lipid packing densities in the membrane rather than by a change of the charges of the lipid head-groups. Since both the mitochondrial inner membranes and the mitochondrial outer membranes contain about 20% negatively charged lipids [31,32], the more tightly bound and more deeply inserted state of association of the signal sequence of the cytochrome *c* oxidase subunit IV may have an important function *in vivo*.

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