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Preferential lipid association and mode of penetration of apocytochrome *c* in mixed model membranes as monitored by tryptophanyl fluorescence quenching using brominated phospholipids

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The fluorescence of the single tryptophan residue at position 59 in apocytochrome *c*, the biosynthetic precursor of the inner mitochondrial membrane protein cytochrome *c*, was studied in small unilamellar vesicles composed of phosphatidylserine (PS) and phosphatidylcholine (PC) with or without specifically Br-labelled acyl chains at the *sn*-2 position. The protein has a very high affinity for PS-containing vesicles (dissociation constant $K_d < 1$ μM). From the relative quenching efficiency by the brominated phospholipids, it could be concluded that the protein specifically associates with the PS component in mixed vesicles and that maximal quenching occurred with phospholipids in which the bromine was present at the 6,7-position of the 2-acyl chain suggesting that (part of) the bound protein penetrates 7–8 Å deep into the hydrophobic core of the bilayer.

Introduction

Apocytochrome *c* belongs to the large group of cytoplasmatically synthesized mitochondrial precursor proteins [1,2]. To reach its final submitochondrial localization (the outside of the innermembrane) the protein must be translocated across the outer mitochondrial membrane and subsequently covalent heme coupling is required [3,4]. Due to the fact that apocytochrome *c* can be chemically prepared in large amounts, it is a suitable model protein to study lipid–precursor protein interactions in order to get insight into the involvement and importance of this interaction in protein translocation across membranes. In previous studies [5,6], we showed that due to its basic character, apocytochrome *c* binds to model membranes, containing negatively charged phospholipids. This primarily electrostatic interaction is attended by a deep penetration of the protein into the hydrophobic core of the lipid bilayer [7,8], which even can result in (partial) translocation of the peptide [9,10]. This deep penetration is specific for the apoprotein and does not occur for the haem containing holocytochrome *c* [8,28]. In the present study we try, by using phospholipids which are brominated at different positions in the fatty

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Abbreviations: LUV, large unilamellar vesicles; Pipes, 1,4-piperazinediethanesulfonic acid; 2-Br-PC, 1-palmitoyl-2(2-bromohexadecanoyl)-PC; 6,7-PC, 1-palmitoyl-2-petroselinoyl-PC; 11,12-PC, 1-palmitoyl-2-vaccenoyl-PC; 6,7-Br-PC, brominated 6,7-PC; 11,12-Br-PC, brominated 11,12-PC.

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acyl chain, to get more insight into the depth of bilayer penetration of the tryptophan-containing part of apocytochrome *c* and the preference of lipid–protein interactions in mixed neutral/negatively charged lipid model membranes. Brominated lipids are able to reduce the fluorescence of tryptophan in proteins by means of a static quenching process [11,12], which requires close contact (≈ 5 Å) between fluorophore and quencher.

Experimental

Bovine brain PS and egg-yolk pC were isolated and purified as described before [15,16]. 1-palmitoyl-lysoPC (lysoPC) was prepared from DPPC (synthesized according to Ref. 17) by treatment with phospholipase A_2 from *Crotalis adamanteus* [18]. *cis*-Vaccenic acid (18:1, Δ11) and *cis*-petroselinic acid (18 : 1, Δ6) were purchased from NU-Chek Prep. Inc. (Elysian, MI, U.S.A.) and 2-bromohexadecanoic acid from Jansen Chimica (Beerse, Belgium). 1-Palmitoyl-2-(2-bromohexadecanoyl)-PC (2-Br-PC), 1-palmitoyl-2-petroselinoyl-PC (6,7-PC) and 1-palmitoyl-2-vaccenoyl-PC (11,12-PC) were prepared by acylation of lysoPC with the fatty acid anhydrids according to Gupta et al. [19]. 6,7-PC and 11,12-PC were brominated at −20°C in chloroform to yield 6,7-Br-PC and 11,12-Br-PC as described in Refs. 13 and 20. The corresponding brominated PS species were prepared by phospholipase D mediated base exchange to yield 6,7-Br-PS and 11,12-Br-PS [21]. It was not possible to obtain 2-Br-PS by this method. 6,7-Br-PS and 11,12-Br-PS were purified by high pressure liquid chromatography [15]. The brominated phospholipids were examined by GLC-mass spectrometry and proton NMR to confirm the identity of the compounds and were found to be pure by thin-layer chromatography. Apocytochrome *c* was prepared from cytochrome *c* of horse heart (Sigma, St. Louis, MO, USA) as described [22]. After renaturation the protein was stored at −20°C in 100 mM NaCl, 10 mM 1,4-piperazinediethanesulfonic acid (Pipes buffer) (pH 7.0) at a concentration of 1.25 mg/ml. Small unilamellar vesicles (SUV) of phospholipid mixtures were prepared by sonication of a dispersion of 2 μmol phospholipid in 1 ml Pipes buffer using a Branson B-12 sonifier (medium tip) under continuous flow of nitrogen at 0°C for 5 min followed by centrifugation at $27000 \times g$ for 20 min in order to remove titanium and multilamellar structures. Tryptophan fluorescence was measured at 30°C on a Perkin-Elmer LS-5 fluorimeter by excitation at 295 nm (slit 10 nm) and recording the emission spectrum between 320 and 360 nm (slit 10 nm). Apocytochrome *c* (50 μg/ml) was titrated by stepwise addition of phospholipid vesicles composed of PS/PC (1 : 1, mol/mol), PS/PC (7 : 3, mol/mol) or pure PS. As a binding model was used $L_n + P \rightleftharpoons PL_n$, where [P] is the free protein concentration, $[L_n]$ is the free lipid concentration divided by the number of lipid-binding sites *n* on the protein and $[PL_n]$ is the concentration of the lipid-protein complex. The dissociation constant K_d is equal to $K_d = [P] \cdot [L_n]/[PL_n]$.

The K_d values were calculated from the binding curves by using the iterative non-linear regression methods as described by Hille et al. [23].

Results and Discussion

Binding of apocytochrome *c* to negatively charged phospholipid SUV is accompanied by an increase of the fluorescence of the single tryptophan residue and a blue shift from 347 to 340 nm. We first characterized this fluorescence increase for the small mixed PC-PS vesicles we used in this study [7]. Fig. 1 shows a typical titration experiment of apocytochrome *c* (4 μM) with vesicles of pure PS, PS/PC (7 : 3, mol/mol) and PS/PC (1 : 1, mol/mol), respectively. Since the relative increase in fluorescence is related to protein binding to the vesicles it can be concluded that the extent of protein binding is related to the PS content of the vesicles. The binding data derived from these titrations are presented in Table I. The low K_d values, which slightly increase with the PS content, demonstrate the very efficient binding of the protein to the PS component in these SUV. A comparable value for the $K_d = 0.05$ μM was reported for the binding of apocytochrome *c* to the putative receptor on the mitochondrial outer membrane [24]. This raises the interesting possibility that the negatively charged lipid component of the outer mitochondrial mem-

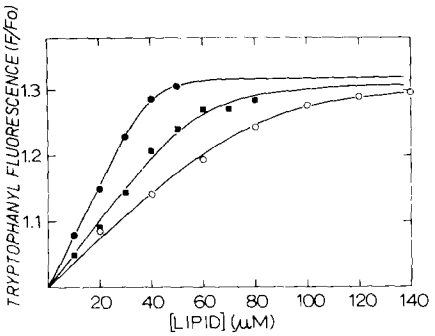

Fig. 1. Binding of apocytochrome *c* (4 μM, 30°C, pH 7.0) to SUV of different mixtures of PC and PS as monitored by the increase of tryptophanyl fluorescence: 100% PS (●), PS/PC (7:3, mol/mol; ■); PS/PC (1:1, mol/mol; ○). The computer drawn curves are obtained from the data points as described in Materials and Methods.

brane is the primary binding site for apocytochrome *c*. Similar titration curves with large unilamellar vesicles (LUV), prepared by the reverse phase evaporation method [25], yielded sigmoidal titration curves from which it was impossible to derive reliable binding data. However, if we compare the present results with binding constants obtained from experiments in which the vesicle bound protein was pelleted and the unbound protein was quantified via a protein determination (K_d = 15 μM for pure PS vesicles; ref. 6), it can be suggested that the apocytochrome *c* binding to SUV is more efficient than to LUV. That an increased bilayer curvature promotes protein-vesicle binding was previously reported for apo A-I [26] and the PC transfer protein [27]. It should be noted that the apocytochrome *c*–PS interaction does not result in vesicle fusion [6].

The low K_d for apocytochrome *c* binding to the SUV makes these vesicles particularly useful for fluorescence studies since nearly complete binding can be obtained at relatively low lipid concentrations. In agreement with data obtained with LUV [6], about 10 PS molecules bind electrostatically, nearly stoichiometrically, per apocytochrome *c* molecule (net positive charge + 9 at neutral pH), independent of the PS fraction (Table I).

Insight in both the depth of penetration of the tryptophan residue and a possible preferential interaction with PS can be obtained from quenching experiments using the different brominated lipids present in mixed PC/PS (1:1, mol/mol) SUV. In the typical quenching experiment shown in Fig. 2, the protein was titrated with vesicles containing the brominated PC (Fig. 2A) or brominated PS (Fig. 2B). From the comparison with the non-brominated (1:1, mol/mol) PS/PC vesicles, it can be concluded that maximal quenching of the tryptophanyl fluorescence occurred with the vesicles containing 6,7-Br-PS. The efficiency of fluorescence quenching by the brominated lipids follows the order: 6,7-Br-PS > 11,12-Br-PS > 6,7-Br-PC > 11,12-Br-PC > 2-Br-PC.

Two conclusions can be drawn from these data. Firstly, the tryptophan of (part of) the bound apocytochrome *c* is preferentially localized near position 6 and 7 of the acyl chain at the 2-position

TABLE I

DISSOCIATION CONSTANT OF APOCYTOCHROME *c* BINDING TO SUV OF PS AND PS/PC MIXTURES

The data presented here are derived from the measured points in Fig. 1. The errors represent the accuracy for the best computer fit of the data points. Similar values of K_d and n were found for different experiments. n is the number of lipid-binding sites

Lipid	K_d (M)	n
PS	0.04 ± 18%	10.2 ± 1.1%
PS/PC (7:3, mol/mol)	0.19 ± 27%	14.5 ± 4.3%
PS/PC (1:1, mol/mol)	0.25 ± 20%	20.4 ± 3.9%

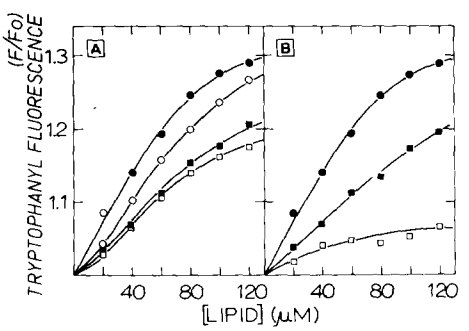

Fig. 2. Quenching of apocytochrome *c* (4 μM) tryptophanyl fluorescence with brominated phospholipids in SUV containing (1:1, mol/mol) mixtures of: (A): PS/PC (control, ●); PS/2-Br-PC (○); PS/6,7-Br-PC (□); PS/11,12-Br-PC (■); and mixtures of: (B): PS/PC (control, ●); 6,7-Br-PS/PC (□) and 11,12-Br-PS/PC (■). Experimental conditions as in Fig. 1.

of the phospholipid molecule. If we assume that this is the result of penetration of the protein into the bilayer then this corresponds to a depth of penetration of about 7–8 Å from the polar headgroup into the hydrophobic core of the bilayer. We cannot exclude the theoretical possibility that the lipid is pulled out of the bilayer by the protein in the protein-vesicle complex. Secondly, at the level of this penetrated tryptophan residue a preferential interaction occurs with the PS component, since the 6,7-Br-PC in the mixed vesicles shows less quenching. The selective enrichment of the PS component around the protein might be of importance for the formation of a translocation site in the membrane. It is furthermore of interest to note that the absolute quenching efficiency is relatively low (maximally 20% for 6,7-Br-PS) which for tryptophan in other proteins with similar lipids can be as high as 100% [13,14]. This might suggest that the protein can adopt different conformations in the vesicle-associated form. A surface location of part of the tryptophan-containing part of the protein, was suggested by tryptophan fluorescence quenching with aqueous quenchers [7]. Furthermore, it is known that the protein can oligomerize especially in the present of lipid [6]. In such protein oligomers protein–protein interactions could shield the tryptophan from the hydrophobic lipid matrix which could contribute to the relatively low quenching efficiency.

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