Volume 261, number 1, 55–58 FEBS 08103 February 1990

Phenethyl alcohol disorders phospholipid acyl chains and promotes translocation of the mitochondrial precursor protein apocytochrome *c* across a lipid bilayer

Wilco Jordi°, Ria Nibbeling° and Ben de Kruijff°+

°*Centre for Biomembranes and Lipid Enzymology* and +*Institute of Molecular Biology and Medical Biotechnology, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands*

Received 18 December 1989

The interaction of phenethyl alcohol with model membranes and its effect on translocation of the chemically prepared mitochondrial precursor protein apocytochrome *c* across a lipid bilayer was studied. Phenethyl alcohol efficiently penetrates into monolayers and causes acyl chain disordering judged from deuterium nuclear magnetic resonance measurements with specific acyl chain-deuterated phospholipids. Translocation of apocytochrome *c* across a phospoholipid bilayer was stimulated on addition of phenethyl alcohol indicating that the efficiency of translocation of this precursor protein is enhanced due to a disorder of the acyl chain region of the bilayer.

Apocytochrome *c*; Deuterium nuclear magnetic resonance; Protein-lipid interactions; Mitochondrial protein import; Phenethyl alcohol

1. INTRODUCTION

It is well known that drugs which interact with lipids influence translocation of proteins across biological membranes [1]. A well-studied lipid-interacting drug is phenethyl alcohol, for which it was recently reported that protein translocation is stimulated in the presence of 1% (v/v) phenethyl alcohol and even allows translocation of translocation-incompetent mature proteins [2]. However, experiments with biological membranes cannot discriminate between a direct or indirect involvement of phospholipids in protein translocation pathways. In this study, we have analyzed the effect of phenethyl alcohol on model membranes by monolayer techniques and by [^{2}H]NMR measurements on specifically deuterated liquid-crystalline, unsaturated phospholipid model membranes. In addition, we have investigated the effect of phenethyl alcohol on translocation of apocytochrome *c* across a lipid bilayer. The mitochondrial precursor protein apocytochrome *c*, follows a relatively simple import route into the mitochondrion, directly across the outer mitochondrial membrane into the intermembrane space, involving the cytochrome *c* heme lyase enzyme [3,4]. The translocation of apocytochrome *c* across the outer mitochondrial membrane could be closely mimicked in a pure lipid bilayer [5]. We show in this study that phenethyl alcohol disorders the acyl chain region of the bilayer and promotes (partial) translocation of the precursor across the bilayer. This observation supports but does not prove the viewpoint that the translocation-promoting effects in more complex membrane systems are a result of the direct interaction of precursor proteins with a more 'fluid' bilayer.

Correspondence address: W. Jordi, Centre for Biomembranes and Lipid Enzymology, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

Abbreviations: [^{2}H]NMR, deuterium nuclear magnetic resonance; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; Av_q, quadrupolar splitting; PIPES, 1,4 piperazindiethane sulfonic acid; PEA, 2-phenylethanol or phenethyl alcohol

2. EXPERIMENTAL

PEA was from Merck (Darmstadt, FRG) and was used without further purification.

DOPC, DOPE and DOPS were synthesized and purified according to established methods [6,7]. Beef heart cardiolipin was purified as published [8]. Cholesterol was from Merck (Darmstadt, FRG). Acyl chain-deuterated (1,2-[11,11-$^2H_2$]dioleoylphosphatidylcholine was synthesized essentially as previously published [9].

Apocytochrome *c* was prepared by removal of the heme group from cytochrome *c* [10] and was subsequently subjected to a renaturation procedure as described [11]. The protein was stored at −20°C in 50 mM NaCl, 10 mM PIPES, 1 mM EDTA, pH 7.0, 0.01% β-mercaptoethanol (PIPES buffer) at a concentration of approximately 1.5 mg/ml as determined using the molar extinction coefficient as previously reported [12].

Monolayer experiments were performed at 30°C at a constant area as previously described [13].

Samples for NMR were prepared by dispersing 40 μmol of lipid (dried overnight under high vacuum) in 1 ml of deuterium-depleted PIPES buffer at room temperature and subsequently the dispersion was subjected to five freeze-thaw cycles after which minor changes in pH (<1 unit) were adjusted with a deuterium-depleted NaOH solution. The dispersion was transferred into a 10-mm NMR tube. PEA was added directly in the NMR tube from a 10% (v/v) stock solution

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

in deuterium-depleted PIPES buffer and incubated for 5 min prior to the NMR measurement. Upon prolonged incubation, no further spectral changes took place.

[^{2}H]NMR spectra were recorded on the MSL 300 at 46.1 MHz with a 11 μs 90° radiofrequency pulse. A quadrupolar echo pulse sequence was used as described [9] with a pulse separation of 35 μs, a 71.5 kHz sweep width, 4 K data points, and a 0.2-s interpulse time. An exponential multiplication (corresponding with 200 Hz line broadening) was applied to the accumulated free induction decays before Fourier transformation and the spectra were subsequently symmetrized.

Proton-noise decoupled [^{31}P]NMR spectra were recorded at 121 MHz on the above spectrometer with a 14 μs 90° radiofrequency pulse and a 1 s interpulse time. The NMR experiments were performed at 0°C and 30°C.

Translocation experiments and control experiments were performed essentially as previously described [5] with two major improvements: (1) vesicles were prepared by extrusion techniques using filters with 400 nm pore size as previously described [14] which eliminated the use of organic solvents; and (2) the trypsin concentration inside the vesicles was decreased from 2 to 0.2 mg/ml, which decreases a potential influence of trypsin on the translocation of apocytochrome *c*.

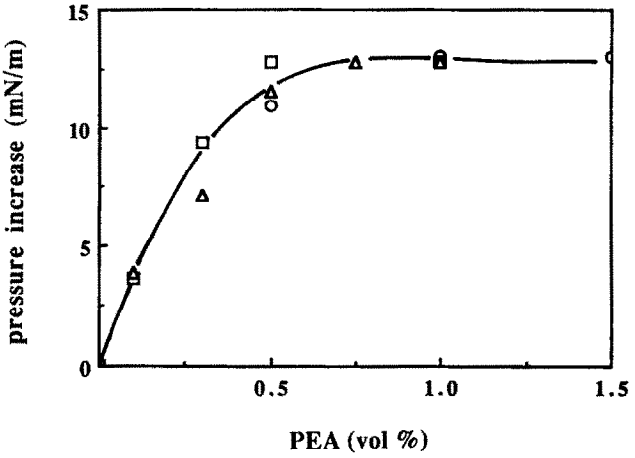

Fig.1. Interaction of phenethyl alcohol with monolayers of DOPS (□), DOPC (△) and an equimolar mixture of both phospholipids (○) at an initial surface pressure of 30 mM/m.

3. RESULTS AND DISCUSSION

To get insight into the interaction of PEA with membranes and its translocation promoting effects, we have investigated the interaction of the drug with model membranes by monolayer techniques and [^{2}H]NMR measurements. In addition, we have studied the effect of PEA on translocation of the mitochondrial precursor protein apocytochrome *c* across a lipid bilayer.

Efficient insertion of the precursor protein into model membranes requires both negatively charged phospholipids and a bilayer in the liquid-crystalline state [5]. Therefore, lipid dispersions of an equimolar mixture of DOPS and DOPC were chosen as test model systems.

Injection of PEA underneath monolayers composed of DOPS and DOPC (molar ratio 1:1) results in large increases in surface pressure approaching the collapse pressure of a lipid monolayer at approximately 0.5% (v/v) PEA (fig.1). This indicates that the drug efficiently penetrates into the monolayer. Identical results are obtained with monolayers composed of either the negatively charged DOPS or the zwitterionic DOPC (fig.1), suggesting that the large increases in surface pressure are caused by hydrophobic interactions between the phenyl group and the monolayer.

The effect of PEA on the order of the phospholipid head group and acyl chains was investigated with a lipid dispersion of an equimolar mixture of DOPS and DOPC, in which the DOPC molecule was specifically labeled with two deuterons at the 11-position of both acyl chains. The residual chemical shift anisotropy of the [^{31}P]NMR spectra, which is a measure for the order in the phosphate region of the polar head group, remained unchanged up to 2% (v/v) PEA (data not shown), indicating that the overall bilayer organization is maintained and that PEA does not affect the order of the lipid head group in this concentration range. In strong contrast, in [^{2}H]NMR spectra, the peak separation or residual quadrupolar splitting ($\Delta\nu_q$) dramatically decreases from 5.9 kHz in the absence of 2.9 kHz in the presence of 1% (v/v) PEA (fig.2). The residual quadrupolar splitting of the [^{2}H]NMR spectra decreases in a concentration-dependent way (fig.3) and the spectrum converts into an isotropic peak at 2% (v/v) PEA (data not shown). In addition, it is obvious that PEA affects all lipid molecules to the same extent, since only one quadrupolar splitting is observed in [^{2}H]NMR spectra.

$\Delta\nu_q$ is directly related to the orientational order

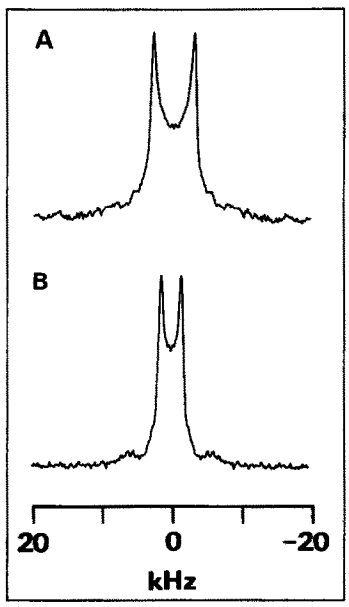

Fig.2. 46.1 MHz [^{2}H]NMR spectra of a dispersion of DOPS:(1, 2-[11,11-$^2H_2$] DOPC (molar ratio 1:1) in the absence (A) and presence (B) of 1% (v/v) phenethyl alcohol at 30°C.

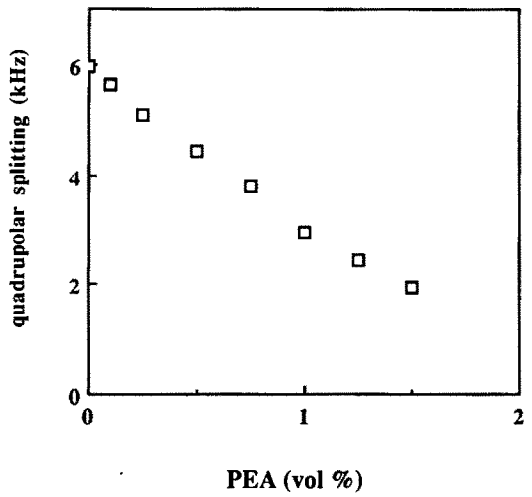

Fig.3. Effect of phenethyl alcohol at 30°C on the quadrupolar splitting of 46.1 MHz [^{2}H]NMR spectra of a dispersion of equimolar mixtures of DOPS and DOPC with the deuterons attached to the fatty acyl chains of the DOPC molecules.

parameter of the C-^{2}H bond ($S_{C\text{-}^2H}$) through the equation [15,16]:

$$\Delta\nu_q = \frac{3}{4}\left(\frac{e^2q\,Q}{h}\right) \cdot S_{C\text{-}^2H}, \qquad (1)$$

where ($e^2q\,Q/h$) is the static quadrupolar coupling constant (170 kHz for a C-^{2}H bond).

From this equation, the value for the order parameter decreases from 0.046 in the absence to 0.023 in the presence of 1% (v/v) PEA at 30°C. In this paper, we will use 'order' and 'disorder' to describe the lipid organization judged from the quadrupolar splitting of the [^{2}H]NMR spectra. It is important to notice that the order parameter calculated from [^{2}H]NMR spectra contains contributions from both the average orientation of the C-^{2}H bond with respect to the axis of motional averaging and the amplitude of this motion. The dramatic decrease in order parameter clearly shows that PEA strongly perturbs the acyl chain organization, in agreement with experiments with an ESR probe [1]. Ethylene glycol at much higher concentrations has similar effects [17]. Hydrophobic alcohols are thought to be anchored with the hydroxyl group in the head group-acyl chain interface of the bilayer [18]. Assuming this orientation, the relatively small hydrophobic phenyl group of PEA will only partially penetrate the bilayer. We suggest that as a result of spacing of the phospholipid head groups, the motional freedom of the deuterons at position-11 of the acyl chains is increased.

To get insight into the effect of PEA on translocation of a precursor protein across a lipid bilayer, we studied the digestion of the mitochondrial precursor protein apocytochrome *c* by trypsin enclosed in large unilamellar vesicles, as previously described [5], in the absence and presence of 1% (v/v) PEA. This concentration was chosen because it was shown to promote protein translocation in an E. coli in vitro translocation system [2]. Fig.4A shows that also in the improved assay used here, apocytochrome *c* is rapidly degraded by trypsin enclosed in the DOPS:DOPC (molar ratio 1:1) vesicles at 30°C, indicating the a part of the protein has penetrated to the opposite interface where it can be digested by trypsin. In vesicles composed of a lipid mixture of DOPC:DOPE:DOPS:cardiolipin:cholesterol (molar ratio 5:3:1:0.5:0.3) which mimics the lipid composition of the outer mitochondrial membrane of rat liver mitochondria [19], apocytochrome *c* is also able to reach the opposite interface. However, the rate of translocation is decreased compared to the DOPS:DOPC (molar ratio 1:1) vesicles, in agreement with the importance of the percentage of negatively charged phospholipid for translocation of apocytochrome *c* across a lipid bilayer [5]. For both lipid mixtures the digestion of apocytochrome *c* is more rapid in the presence of 1% (v/v) PEA, indicating that the protein 'translocates' faster. For the DOPS:DOPC (molar ratio 1:1) and DOPC:DOPE:DOPS:cardiolipin:cholesterol (molar ratio 5:3:1:0.5:0.3) vesicles, the half lifetime, $t_{1/2}$, of apocytochrome *c* translocation decreases from 20 and 46 min in the absence to 6 and 23 min in the presence of 1% (v/v) PEA, respectively (fig.4). This two- to three-

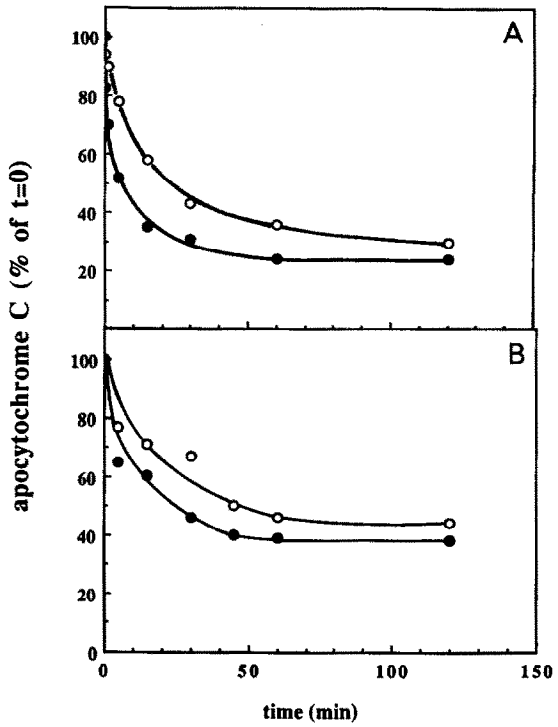

Fig.4. Digestion of apocytochrome *c* by trypsin enclosed in (A) DOPS:DOPC (molar ratio 1:1) and (B) DOPC:DOPE:DOPS:cardiolipin:cholesterol (molar ratio 5:3:1:0.5:0.3) vesicles at 30°C in the absence (○) and presence (●) of 1% (v/v) phenethyl alcohol. Average of two independent experiments, average deviation from the mean is 4% of the amount of apocytochrome *c* at $t=0$.

fold reduction in $t_{1/12}$ is not due to leakage of trypsin out of the vesicles or a direct stimulatory effect of PEA on trypsin activity (data not shown). The protease sensitivity of apocytochrome *c* for trypsin in solution or trypsin externally bound to vesicles was identical in the absence and presence of 1% (v/v) PEA, indicating that the conformation of apocytochrome *c* was similar under both conditions (data not shown).

Previous [^{2}H]NMR and translocation experiments with apocytochrome *c* showed that cholesterol and a low temperature, conditions which cause an increase in order of the fatty acids of the lipids judged from the quadrupolar splitting of the [^{2}H]NMR spectra [20], also decrease the rate of translocation of apocytochrome *c* in model membrane experiments [5,21]. In addition, it has been shown that import of various mitochondrial precursor proteins into mitochondria is inhibited at lower temperatures (for review see [22]). The stimulation of translocation in a 'translocation assay' which only contains a pure phospholipid bilayer and a chemically prepared precursor protein, suggests that stimulation of translocation in more complex biological membranes by phenethyl alcohol is a direct result of the interaction of precursor proteins with a more 'fluid' bilayer. If such a translocation pathway directly through the lipid bilayer is part of the normal route or a 'by-pass' pathway created by an increased disorder of the acyl chain region remains to be investigated.

Acknowledgements: R.A. Demel is gratefully acknowledged for expert assistance is monolayer measurements. This work was carried out under the auspices of the Netherlands Foundation of Chemical Research (SON) and with the financial aid from the Netherlands Organization for Scientic Research (NWO).

REFERENCES

[1] Halegoua, S. and Inouye, M. (1979) J. Mol. Biol. 130, 39–61.
[2] Chen, L. and Tai, P.C. (1987) J. Bacteriol. 169, 2373–2379.
[3] Dumont, M.E., Ernst, J.F. and Sherman, F. (1988) J. Biol. Chem. 263, 15928–15937.
[4] Nicholson, D.W., Hergersberg, C. and Neupert, W. (1988) J. Biol. Chem. 263, 19034–19042.
[5] Rietveld, A., Jordi, W. and De Kruijff, B. (1986) J. Biol. Chem. 261, 3846–3856.
[6] Comfurius, P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta 488, 36–42.
[7] Van Deenen, L.L.M. and De Haas, G.H. (1964) Adv. Lipid Res. 2, 168–229.
[8] Smaal, E.B., Romijn, D., Geurts Van Kessel, W.S.M., De Kruijff, B. and De Gier, J. (1985) J. Lipid Res. 26, 633–637.
[9] Chupin, V., Killian, J.A. and De Kruijff, B. (1987) Biophys. J. 51, 395–405.
[10] Fisher, W.R., Taniuchi, H. and Anfinsin, C.B. (1973) J. Biol. Chem. 248, 3188–3195.
[11] Hennig, B. and Neupert, W. (1983) Methods Enzymol. 97, 261–274.
[12] Stellwagen, E., Rysavy, R. and Babul, G. (1972) J. Biol. Chem. 247, 8074–8077.
[13] Demel, R.A., Kalsbeek, R., Wirtz, K.W.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 466, 10–22.
[14] Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1983) Biochim. Biophys. Acta 812, 55–65.
[15] Seelig, J. (1977) Quart. Rev. Biophys. 10, 353–418.
[16] Davis, J.H. (1983) Biochim. Biophys. Acta 737, 117–171.
[17] Nicolay, K., Smaal, E.B. and De Kruijff, B. (1986) FEBS Lett. 209, 33–36.
[18] Pope, J.M., Walker, L.W. and Dubro, D. (1984) Chem. Phys. Lipids 35, 259–277.
[19] Hovius, R., Lambrechts, H., Nicolay, K. and De Kruijff, B., Biochim. Biophys. Acta., in press.
[20] Gasset, M., Killian, J.A., Tournois, H. and De Kruijff, B. (1988) Biochim. Biophys. Acta 939, 79–88.
[21] Zhou, L.X., Jordi, W. and De Kruijff, B. (1988) Biochim. Biophys. Acta, 942, 115–124.
[22] Hartl, F.-U., Pfanner, N., Nicholson, D.W. and Neupert, W. (1989) Biochim. Biophys. Acta 988, 1–45.