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Magic angle spinning ^{13}C-NMR spin-lattice relaxation study of the location and effects of α-tocopherol, ubiquinone-10 and ubiquinol-10 in unsonicated model membranes

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Abstract. α-Tocopherol, ubiquinone-10 and ubiquinol-10 have been studied by high resolution magic angle samples spinning ^{13}C-nuclear magnetic resonance in egg yolk phosphatidylcholine multilamellar vesicles model membranes in order to assess their location and the induced perturbations on this model system. α-Tocopherol is placed in such a position that it is in close contact with the head group of the phospholipid and exposed to the solvent. In this position it significantly perturbs the phospholipid head group, the 5a-CH_3 and the 7a-CH_3 groups being the closest parts to the membrane surface. On the other hand, ubiquinol-10 perturbs the membrane surface more than ubiquinone-10, but neither compound significantly changed the phospholipid head group conformation.

Key words: α-Tocopherol – Ubiquinol-10 – Ubiquinone-10 – Magic angle spinning NMR

Introduction

Tocopherols and ubiquinones are terpenoid molecules which are known to be very important components of biological membranes. Tocopherols, of which α-tocopherol (αT) is the most important component (Burton and Ingold 1986), act as potent chain-breaking antioxidants of biological membranes and act against free radical damage (DeDuve et al. 1978). On the other hand, ubiquinones are essential intermediates in the respiratory chain of mitochondria and bacteria, being alternatively oxidized and reduced (Trumpower 1981; Lenaz and Fato 1986). In mitochondria, the main species found are ubiquinone-10 and ubiquinol-10 i.e., with ten isoprene units in the long side chain.

In order to better understand the molecular mechanism of action of these molecules, it is important to study their location and organization in the membrane and also their interaction with membrane components. A number of studies have been done on the interaction and location of αT and ubiquinones with lipid membranes, suggesting that αT has its phenolic group located near the polar moiety of the lipid matrix (Villalaín et al. 1986; Perly et al. 1985; Ekiel et al. 1988), whereas the quinone ring of ubiquinones should be buried in the hydrophobic matrix of the bilayer, ubiquinol-10 being nearer the membrane surface than ubiquinone-10 (Michaelis and Moore 1985; Ulrich et al. 1985).

Nuclear magnetic resonance (NMR) spectroscopy has been used extensively to investigate the structure and dynamics of model and biological membranes but, unfortunately, conventional NMR spectra of intact membranes are basically uninformative. Recently, the application of magic angle sample spinning (MAS) NMR to model and natural membrane systems yielded very highly resolved spectra, enabling detailed studies of their structure and dynamics (Oldfield et al. 1987; Hamilton et al. 1991; Abebodun et al. 1992).

The aim of this work is to ascertain in a direct way the location and properties of αT, ubiquinone-10 and ubiquinol-10 inside model multilamellar vesicles (MLV) by the application of MAS ^{13}C-NMR. Information on their effect and location has been obtained from the variation of spin-lattice relaxation times (T_1) of both the phospholipid and terpenoid molecules in the absence and in the presence of a paramagnetic agent, Gd^{3+}. The effect of Gd^{3+} on the spin-lattice relaxation times of the lipid resonances has an explicit distance dependence, allowing it to be used to evaluate relative distances on a molecular scale.

Material and methods

Egg yolk phosphatidylcholine (EYL) was obtained from Lipid Products (Surrey, Great Britain), ubiquinone-10 and αT were obtained from Sigma (Madrid, Spain) and $GDCl_3 \cdot 6H_2O$ from Aldrich (Madrid, Spain). All other reagents used were of analytical grade. Water was twice

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distilled and deionized in a Milli-Q apparatus from Millipore. The purity of EYL before and after the measurements was checked by thin-layer chromatography and showed only one spot.

Sample preparation

Ubiquinol-10 was obtained from ubiquinone-10 as described by Rieske (1976). The assay of ubiquinone-10, ubiquinol-10 and αT in solution was done by ultraviolet spectrophotometry as described by Crane and Barr (1970) for ubiquinones and by Villalaín et al. (1986) for αT.

The samples contained 200 mg of EYL in chloroform and, if required, the appropriate amounts of ubiquinone-10 and ubiquinol-10 or αT in chloroform:methanol 1:1 (v/v) in order to obtain a phospholipid to terpenoid ratio of 3:1. After drying under a stream of O_2-free N_2 in the dark, the samples were then further desiccated under high vacuum in the dark for more than eight hours in order to remove the last traces of solvent. The samples were dispersed and hydrated in 500 µl H_2O, MLV were formed by careful using a bench vibrator and kept for 30 min at 50 °C, with occasional mixing in a vortex mixer in order to obtain a homogeneous and uniform suspension. When required, MLV were formed in the presence of two different final concentrations of Gd^{3+}, 5×10^{-3} and 10^{-2} M. To assure sample homogeneity, freezing, thawing and vortexing were applied twice to the samples. Subsequently the samples were spun and pelleted at high speed in a bench microfuge.

Nuclear magnetic resonance

Magic angle spinning (MAS) ^{13}C-NMR spectra were obtained in the Fourier transform mode on a Varian Unity 300 spectrometer, operating at 75.429 MHz for carbon, using a Jakobsen 7 mm room temperature CP/MAS probe from Varian. ^{13}C MAS experiments (dipolar decoupled during data acquisition, 100 W) were recorded using (typically) 8 µs ^{13}C 90° pulse widths and spinning rates of ~1–1.2 KHz (Abebodun et al. 1992). The number of scans for each delay was typically 1 000. All spectra were referenced with respect to an external standard of tetramethylsilane, with high-frequency, low-field, paramagnetic or deshielded values being denotes as positive (IUPAC δ scale) and chemical shifts were reproducible to better than ± 0.1 ppm.

^{13}C-NMR T_1 measurements were carried out by the fast inversion-recovery method (Canet et al. 1975) employing a minimum of seventeen different delay values. T_1 values were obtained from a non-linear least-squares fit to $M_\tau = M_\infty(1 - Ne^{-\tau/T_1})$, where M_∞ is the thermal magnetization value (M_τ when $\tau = \infty$), M_τ is the magnetization value for the particular τ value employed and N is adjusted by the fitting ($N = 2$). The estimated uncertainty T_1 values arising from this procedure is approximately 15% unless noted otherwise, estimated from replica experiments and scatter within each experiment. Since long

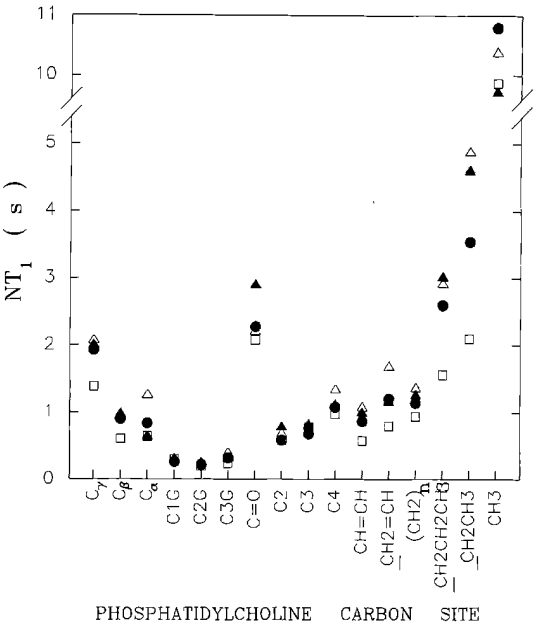


Fig. 1. Carbon-13 NMR spin-lattice relaxation time (NT_1) in second for EYL resonances, either in pure form (●) or in the presence of (□) α-tocopherol, (△) ubiquinone-10 and (▲) ubiquinol-10 at a EYL:terpenoid ratio of 3:1. Phospholipid carbon sites corresponding to the headgroup are marked with a greek letter, those corresponding to the glycerol backbone are marked with the letter G and the unmarked ones belong to the acyl hydrocarbon chain

T_1 experiments are particularly sensitive to long-term changes in magnetic homogeneity, short and long τ values were alternated and interleaved by the spectrometer computer.

Results and discussion

MAS ^{13}C-NMR spectra of MLV formed by either pure EYL or EYL plus either αT, ubiquinone-10 or ubiquinol-10 yielded very well resolved spectra (not shown for brevity) in which four main spectral regions could be discerned: carbonyl groups, olefinic carbons, glycerol backbone carbons and acyl chain aliphatic carbons. Some more resonances, belonging to the terpenoid molecules, could also be observed. The assignment of the different resonances of the phospholipid was made according to Stoffel et al. (1972) and Lee et al. (1974). Because of the highly resolved nature of the MAS ^{13}C-NMR spectra it was possible to determine the T_1 of most individual carbon atom sites of EYL. Since the relaxation rate is proportional to the number of directed bonded hydrogrens (N) the relaxation parameter T_1 has been scaled by the number of attached hydrogen atoms to produce NT_1. NT_1 is plotted in Fig. 1 as a function of the position in the phospolipid. The NT_1 values of EYL in the absence Gd^{3+} were quite similar to other previously reported NT_1 values (Yeagle and Frye 1987).

The NT_1 values of the clycerol backbone carbons of EYL were very similar either in pure EYL or in the presence of either αT, ubiquinone-10 or ubiquinol-10 (Fig. 1). However, the NT_1 of the headgroup and the acyl chain

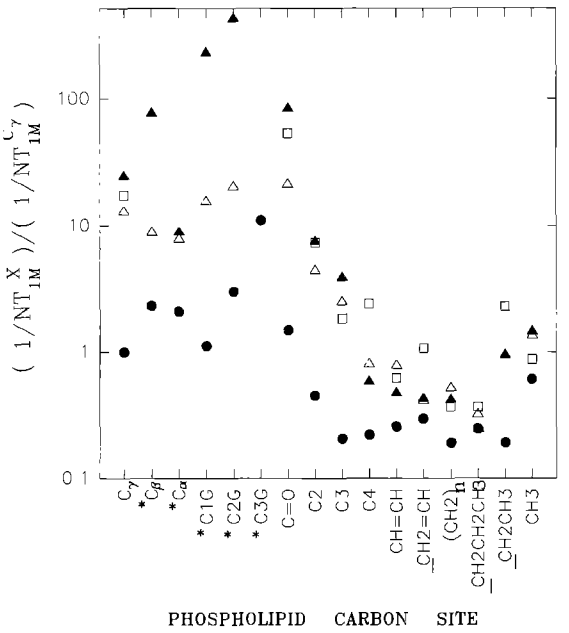


Fig. 2. Logarithmic plot of the paramagnetic contribution to the spin-lattice relaxation time of the EYL carbon sites for pure EYL (●) or EYL in the presence of (□) α-tocopherol, (△) ubiquinone-10 and (▲) ubiquinol-10 at a EYL:terpenoid ratio of 3:1. The abscissa defines the different phospholipid carbon sites as in Fig. 1. Starred phospholipid carbon sites define those carbon sites broadened beyond detection upon α-tocopherol inclusion

carbon atoms of EYL in the presence of α *T* were shorter than in pure EYL, suggesting that these regions were more ordered in the presence of α *T*. On the other hand, both ubiquinone-10 and ubiquinol-10 had a different effect, increasing the NT_1 of the acyl chain carbon atoms of EYL without changing the NT_1 values of the head group carbons (Fig. 1). Consequently, both ubiquinone-10 and ubiquinol-10 disorder the acyl chain moiety of EYL.

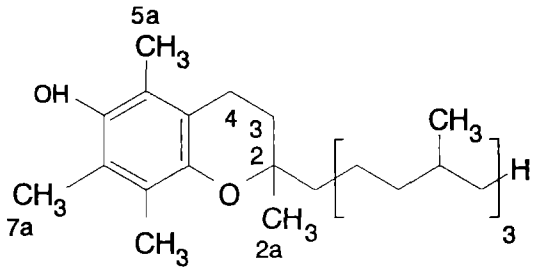
Lanthanide ions, when added to a dispersion of phospholipid vesicles, adsorb to the vesicle surface (Hauser et al. 1975) and cause the phospolipid resonances either to shift or broaden (Bergelson 1978). Gd^{3+}, having relatively long electron relaxation times, results in large perturbations to the T_1 of the nucleus under observation (Dwek 1973). If NT_1^0 is the spin-lattice relaxation time in the absence of Gd^{3+} and NT_1 in its presence, the paramagnetic contribution to the relaxation time is NT_{1M} where $(NT_{1M})^{-1}=(NT_1)^{-1}-(NT_1^0)^{-1}$. This equation should also include a diamagnetic term to allow for any non-paramagnetic influence of binding Gd^{3+}, but it is likely to be negligible (Dwek 1973). In order to obtain information about the relative distance of the different nuclei to the metal ion, the experimentally measured NT_{1M} values were used. According to the Solomon-Bloembergen equation, and considering only dipolar relaxation, we can obtain $(NT_{1M})^{-1}=f(\tau_c)\cdot r^{-6}$, where τ_c is the correlation time for the dipolar interaction and r is the distance between the lanthanide and the nucleus in question (Dwek 1973). Therefore, the ratio of $(NT_{1M})^{-1}$ values of any two nuclei allows the calculation of the ratio of their r values. In this way relative distances could be obtained. However, it has to be taken into account that an essential feature of the Solomon-Bloembergen equation is rapid exchange of the metal, so that the r^6 dependence may not be the only factor involved. In order to circumvent this difficulty we have obtained and compared the relative paramagnetic contribution to the relaxation time of the different EYL carbon sites by taking the ratio of the paramagnetic contribution to the relaxation time of any EYL carbon site in the absence and in the presence of the terpenoid molecules, $(NT_{1M}^X)^{-1}$, and that of the C_γ carbon of pure EYL, $(NT_{1M}^{C_\gamma})^{-1}$.

The relative paramagnetic contribution to the relaxation time of the different EYL carbon sites, either in pure form or in the presence of either α *T*, ubiquinone-10 or ubiquinol-10, is presented in Fig. 2. The most significant effect on the T_1 of pure EYL produced by the presence of Gd^{3+} is found on the C3 glycerol backbone carbon and also, but to a lesser extent, on the C_A headgroup carbon, which is in accordance with previous results which have shown that Gd^{3+} binds to the phosphate group of phospholipids (Hauser et al. 1975). Taking into account the known geometric structure of a phospholipid molecule in the membrane, the C3 glycerol backbone carbon should be the nearest one to the external ion bound to the phosphate group (Small 1986).

The inclusion of α *T*, ubiquinone-10 or ubiquinol-10 in EYL membranes changed dramatically the paramagnetic contribution to the relaxation time of the different EYL carbon atoms (Fig. 2). Some carbons of the phospholipid were so close to the lanthanide ions that they were relaxed to the extent that they were broadened beyond detection. This was the case for the resonances of the head group (except the C_γ), for the glycerol backbone carbons in the presence of α *T*, and for the resonance of the C3 glycerol backbone carbon in the presence of either ubiquinone-10 or ubiquinol-10.

The dramatic effect observed on the T_1 values of the polar phospholipid carbons upon inclusion of α *T* might indicate that the lanthanide ion was capable of entering more deeply into the palisade structure of the membrane and/or that α *T* perturbed the phospholipid, changing its conformation so that the binding of Gd^{3+} was not the same as in pure EYL. At the EYL/α *T* ratio used in this work (3:1), it has been shown that α *T* is completely incorporated in the membrane, but it certainly perturbs the phospholipid (Villalaín et al. 1986). Therefore α *T* must be found near the lipid/water interface, separating the phospholipid molecules. If this were the case, the resonances of α *T* would be relaxed more efficiently because of its proximity to the lanthanide. This is in fact what is seen (see below). The effect of the incorporate of ubiquinone-10 and ubiquinol-10 in the T_1 of EYL indicated that both terpenoid molecules perturbed, although slightly, the phospholipid molecule, as observed previously (Aranda et al. 1986). But, as seen in Fig. 2, ubiquinol-10 would be more perturbing than ubiquinone-10. In summary, the effect producted on EYL by these molecules would be in the order α *T* > ubiquinol-10 > ubiquinone-10.

It has been possible to determine the T_1 of some of the individual carbon atom sites of α *T* when incorporated in the membrane, both in the absence and in the presence of Gd^{3+} (Table 1). The error of the measurements was ap-



SCHEME I

Table 1. ^{13}C-NMR spin lattice relaxation times (NT_1, s) for α-tocopherol resonances in egg yolk lecithin multilamellar vesicles in H_2O

Site[a]	WYL/α-tocopherol 3:1 [Gd^{3+}]		
	0	5×10^{-3} M	10^{-2} M
2	1.0	0.6	nd[b]
2a	1.0	nd	nd
3	1.4	1.1	1.1
4	1.3	1.1	1.1
5a	3.6	0.4	nd
7a	3.9	0.7	0.6

[a] Resonance assignment according to Ekiel et al. (1988) and Urano et al. (1980)
[b] Not determined because of broadening

proximately ±30% owing to the low intensity of the resonances, but nevertheless some useful information could be obtained from them. Some resonances were broadened beyond detection in the presence of Gd^{3+} (5×10^{-3} M), particularly the 2a-CH_3 group (Scheme I), but also the 5a and 2 groups at a greater Gd^{3+} concentration (10^{-2} M), indicating that these resonances were very close to the lanthanide ion. The other resonances of α*T* were relaxed to a different extent. For example, the relative change in the paramagnetic contribution to the T_1 of the 5a and 7a-CH_3 groups was 3.65 ± 1.04 but 0.33 ± 0.17 for the 2, 3 and 4 carbon sizes of α*T* This different effect of the lanthanide could provide an indication for the existence of a certain location of the molecule in the membrane, but this is certainly a limiting situation. However, it would indicate that the nuclei giving resonances that are relaxed so efficiently would be near the nuclei of the phospholipid which sense the same effect from the lanthanide ion, i.e., the head group and the glycerol backbone. These data suggest that both the 5a-CH_3 and 7a-CH_3 groups of α*T* the nearest ones to the membrane surface.

The T_1 for the 5a-CH_3 group measured by us (Table 1) differs from that previously reported (Perly et al. 1985). The use of small unilamellar vesicles instead of MLV or the different phospholipid to α*T* ratios used could be the source of this discrepancy. Nevertheless, distance information relies on the different effect on relaxation times with and without relaxation probes and not in their absolute value. Using specifically labelled α*T*, recent studies have indicated that the 6-OH and 5a-CH_3 groups should be the closest parts to the membrane surface (Perly et al. 1985; Ekiel et al. 1988). The results obtained in this work using MLV would be consistent with this arrangement, i.e., the chromanol polar head of α*T* should be near the membrane surface and the hydrophobic tail should be embedded in the membrane.

It has also been possible to obtain the T_1 of some of the resonances of ubiquinone-10 and ubiquinol-10. In this case the error of the measurements was greater than 40%, because the signals had a low intensity and also because of overlapping with other phospholipid resonances. This prevented us from obtaining by distance information. In any case, no resonances from either ubiquinone-10 or ubiquinol-10 were broadened beyond detection upon lanthanide addition (not shown), indicating that are deeper in the membrane than α*T* and/or that they perturb the phospolipid molecule to a lesser extent.

These results have several physiological implications. The location of the chromanol ring of α*T* in the vicinity of the lipid-water interphase allows it to act as a free radical scavenger of oxidizing agents coming from the outside of the membrane. However, it can be argued that this location does not allow it to act against lipid peroxyl radicals formed in the interior of the membrane. This paradox may be understood if it is realized that the region of the fatty acyl chain affected by peroxidation will become polar enough to be expected to "float" towards the lipid-water interphase (Barclay and Ingold 1981). On the other hand, the different location of ubiquinone-10 and ubiquinol-10 could be explained by their different hydrophilic/lipophilic balance. This may have physiological significance since it may allow a flip-flop movement of ubiquinone-10 and ubiquinol-10, therefore carrying electrons and protons across the membrane, a property which may be associated with the capacity of coenzyme Q_{10} to act as a mediator in electron transport chains.

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