EPR study of β-phosphorylated stable aminoxyl radicals in the presence of liposomes

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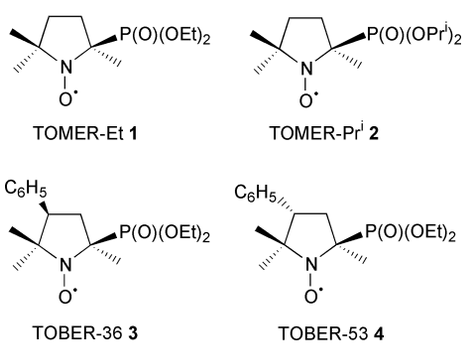
The behaviour of four stable β-phosphorylated aminoxyl radicals of the pyrrolidinoxyl series has been studied by EPR spectroscopy in the presence of dimyristoyl-phosphatidylcholine (DMPC) unilamellar liposomes. The affinity of these compounds for the liposome structure was found to be more or less high depending on their hydrophobicity. In each case, computer simulations of experimental EPR spectra permitted the determination of the hyperfine coupling constants of the radical inserted in the phospholipid bilayer.

Introduction

The potential applications of stable aminoxyl radicals became increasingly important with the observation that these compounds, which have been widely used *in vitro*, could also be employed successfully for various *in vivo* studies. Thus, stable aminoxyl radicals have been used as EPR spin probes in the study of biomolecule dynamics[1] or of membrane properties,[2] in magnetic resonance imagery,[3] in oximetry,[4] or to follow *in vivo* drug delivery *via* liposomes.[5] Such a variety of applications explained the great diversity of stable aminoxyl radicals available, especially as regards their lipophilicity and their EPR parameters. Considering all these biological uses, it could be a major advantage to have a simple physico-chemical technique allowing the location of these radicals in cells to be foreseen before using them *in vivo*. In this field, the study of an aminoxyl radical behaviour in the presence of a membrane model could permit the evaluation of its capacity to enter the cells. In fact, the problem lies in the choice of this model, which must be as simple as possible to be easily implemented, without losing the main characteristics of membranes. Therefore, various organised molecular systems showing a hydrophobic area, such as micelles or vesicles, can be considered as more or less reliable models. From this perspective, we initiated a few years ago EPR studies of the behaviour of stable aminoxyl radicals in the presence of sodium dodecyl sulfate (SDS) micelles.[6] Except in the case of strongly hydrophilic and negatively charged compounds, all the radicals studied were found to exchange between micelles and the bulk aqueous phase approximately at the same rate as an SDS monomer. In the case of β-phosphorylated compounds, the hyperfine coupling constant (hfcc) with the phosphorus was also found to be a good probe to determine the radical environment. However, contrary to membranes, micelles are dynamic structures that do not delimit an internal aqueous cavity, and we felt that the encouraging results obtained could not be directly transposed to cellular media. To proceed with this study, we then focused on the behaviour of these radicals in the presence of dimyristoyl-phosphatidylcholine (DMPC) liposomes, which are considered as a more reliable biological membrane model though they do not reflect their complexity. The first results thus obtained are presented in this paper.

Results and discussion

Four stable β-phosphorylated aminoxyl radicals of the pyrrolidinoxyl series, for which the synthesis has been previously described,[7] have been chosen for this work: 2-diethoxyphosphoryl-2,5,5-trimethyl(pyrrolidin-1-yloxyl) **1** (TOMER-Et), 2-bis(isopropyloxy)phosphoryl-2,5,5-trimethyl(pyrrolidin-1-yloxyl) **2** (TOMER-Pr^i), *r*-2-diethoxyphosphoryl-*c*-4-phenyl-2,5,5-trimethyl(pyrrolidin-1-yloxyl) **3** (TOBER-36) and *r*-2-diethoxyphosphoryl-*t*-4-phenyl-2,5,5-trimethyl(pyrrolidin-1-yloxyl) **4** (TOBER-53).



Kinetic studies of the reduction of these compounds by various biological agents have been performed *in vitro* and acceptable reduction rates have been found.[8] All these pyrrolidinoxyl radicals exhibited EPR spectra showing a main triplet, due to hyperfine coupling of the unpaired electron with the nitrogen nucleus (a_N), split by a large phosphorus hfcc (a_P), very sensitive to the ring conformation.

For each radical, a first series of three reference EPR spectra have been recorded in a pH 7.4 HEPES buffer containing 150 mmol L^{-1} NaCl in the absence of liposomes, and in the presence or in the absence of either ethanol or potassium tris(oxalato) chromate ($K_3Cr(C_2O_4)_3 \cdot 3H_2O$, CROX). The EPR parameters of compounds **1**–**4** obtained are listed in Table 1. In the presence of 50% ethanol, the pyrrolidinoxyl radical EPR spectra showed a weak decrease in both a_N and a_P. This was entirely due to the change to a less polar environment, since we verified that the decrease in the ionic force caused by dilution

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Table 1 EPR parameters in HEPES buffer and in HEPES buffer–ethanol (1 : 1, vol/vol) of the aminoxyl radicals **1**–**4**

	HEPES buffer[a]		HEPES buffer–EtOH[b]	
Aminoxyl	a_N/mT	a_P/mT	a_N/mT	a_P/mT
TOMER-Et **1**	1.53	4.79	1.49	4.79
TOMER-Pr^i **2**	1.54	4.73	1.52	4.72
TOBER-36 **3**	1.52	3.64	1.48	3.60
TOBER-53 **4**	1.53	5.49	1.49	5.45

[a] The HEPES buffer was used at pH 7.4 in the presence of 150 mmol L^{-1} NaCl. [b] pH 7.4 HEPES buffer–EtOH, 1 : 1, vol/vol, in the presence of 75 mmol L^{-1} NaCl.

Table 2 EPR parameters of the aminoxyl radicals **1**–**4** in the aqueous phase and in the liposome phospholipid bilayer and the proportion of aminoxyl inserted into liposomes (R_{lip}) determined by computer simulation of EPR signals recorded in a pH 7.4 HEPES buffer with 150 mmol L^{-1} NaCl in the presence of DMPC unilamellar liposomes (vesicle diameter: 90 nm, final DMPC concentration: 50 g L^{-1})

	Aqueous phase		Liposome bilayer		
Aminoxyl	a_N/mT	a_P/mT	a_N/mT	a_P/mT	R_{lip} (%)
TOMER-Et **1**	1.53	4.79	1.42	4.67	19
TOMER-Pr^i **2**	1.54	4.74	1.43	4.66	41
TOBER-36 **3**	1.52	3.64	1.43	3.49	91
TOBER-53 **4**	1.52	5.48	1.41	5.20	93

with water did not affect hfcc's. Because of the spin–spin relaxation process between the aminoxyl radical and the paramagnetic complex ion CROX, the totality of the radical EPR signal was found to disappear when 50 mmol L^{-1} of CROX were added to the medium.

Then, various spectra were recorded in the presence of unilamellar DMPC liposomes, and two different cases were observed. First, with compounds **2**–**4**, the spectra clearly showed the superposing of two signals, the first with narrow lines and the second with broader lines. In each case, when the liposome structure was totally and irreversibly destroyed by adding 50% ethanol to the medium, a single signal, identical to that recorded in the absence of liposomes, was obtained. On the other hand, the addition of 50 mmol L^{-1} CROX caused the disappearance of the first signal (narrow lines), while the second was still observed. CROX is known to be a strongly hydrophilic and negatively charged relaxing agent ($Cr(C_2O_4)_3^{3-}$), unable to penetrate the phospolipid bilayer. In its presence, only the EPR signal corresponding to the radical in the bulk aqueous phase disappeared, while the spectrum of the radical inserted into the liposomes remained unaffected. It is noteworthy that the signals with broad lines obtained in the presence of CROX always showed significantly lower values for both a_N and a_P than those obtained in pure buffer. For example, in the case of TOBER-36 **3**, the EPR parameters were $a_N = 1.52$ mT and $a_P = 3.64$ mT with linewidths L_{W-1}, L_{W0} and L_{W+1} approximately equal to 0.05 mT in buffer, and $a_N = 1.43$ mT and $a_P = 3.49$ mT with linewidths $L_{W-1} = 0.38$ mT, $L_{W0} = 0.17$ mT and $L_{W+1} = 0.21$ mT in the presence of liposomes and CROX. This a_N decrease indicated that the aminoxyl was located in an environment much less polar than water. The important variation observed in a_P, which reached 0.28 mT in the case of **4**, corresponded to an important change in the main radical conformation. In addition, the line broadening effect could originate from a partial immobilisation of the aminoxyl radical considered. Note however that this could also be the result of a higher oxygen concentration in the apolar environment of the phospholipid bilayer. Anyway, all these results clearly indicated that compounds **2**–**4** partitioned between the bulk aqueous phase and the liposome bilayer, in which their motions were probably restricted. To illustrate these results, the various spectra recorded with TOBER-36 **3** are represented in Fig. 1.

TOMER-Et **1** did not seem to behave the same as **2**–**4**. In this case, the spectrum recorded in the presence of liposomes was found to be almost identical to that obtained in pure buffer, as shown in Fig. 2. However, a weak signal of **1** was still observed in the presence of liposomes after the addition of CROX (see Fig. 2c). Similarly to what was observed with the other radicals, this spectrum showed broader lines than in aqueous media (L_{W-1}, L_{W0}, and L_{W+1} approximately equal to 0.05 mT in buffer and $L_{W-1} = 0.12$ mT, $L_{W0} = 0.07$ mT, and $L_{W+1} = 0.08$ mT in the presence of liposomes and CROX), and probably corresponded to the radical inserted into the liposome bilayer. In addition, the TOMER-Et spectrum recorded in the presence of liposomes was significantly better simulated by considering a second species, *i.e.* **1** inserted in the phospholipid bilayer. Despite this, it appeared that **1** remained essentially in the bulk aqueous phase, since only 20% of this radical was found to enter the liposome structure (see Table 2).

All the various spectra recorded have been simulated using a computer program elaborated by Rockenbauer and Korecz,[9] which permitted the ratio and the EPR parameters of the radicals inserted into the liposomes to be obtained, and the values obtained are collected in Table 2. In each case, the signal obtained in the presence of liposomes could be simulated by adding the simulations of the radical spectra in buffer on one hand, and in the presence of liposomes and CROX on the other hand (see Fig. 1). When the same radicals were previously studied in the presence of micelles,[6] they were found to exchange between water and the micelle structure with an average correlation time of the exchange in the range 3×10^{-7}–9×10^{-7} s.[6] On the contrary, all the spectra recorded in the presence of liposomes could be satisfactorily simulated without introducing an exchange between two paramagnetic species, namely the radical in water and in the phospholipid bilayer. So it appeared that the exchange equilibrium of the radical between water and liposomes was too slow to be detected by conventional EPR spectroscopy. This important difference in the behaviour of **1**–**4** in the presence of micelles or liposomes could be directly linked to the higher stability of the liposome structure.

Generally speaking, the partitioning of compounds **1**–**4** between the liposomes and the surrounding buffer was a slow phenomenon. Actually, an incubation of *ca.* 20 min at 40 °C was necessary to observe the spectra of the radicals inserted into liposomes (see Experimental section). As previously observed in the presence of micelles,[6] the more hydrophobic the compound, the higher was its affinity for the liposomes. However, this phenomenon seems to be less marked than in the case of micelles. Thus, the affinity of TOMER-iPr **2** for the micelles was five times higher than that of TOMER-Et **1**,[6] while the proportion of radical inserted in the liposomes was only two times higher for **2** than for **1**. Note also that the two diastereoisomers **3** and **4** behaved almost the same in the presence of DMPC liposomes, while their affinities for SDS micelles were found to be significantly different. So the radical affinity for the liposomes did not seem to depend on the radical stereochemistry. All these results suggest that the conclusions of our previous study of **1**–**4** in the presence of SDS micelles cannot be directly transposed to liposomes.

Conclusion

This work allowed us to determine hfcc's of stable β-phosphorylated aminoxyl radicals in both an aqueous medium and in a liposome bilayer. As previously observed in the presence of micelles,[6] the hfcc a_P was found to be a good probe of the

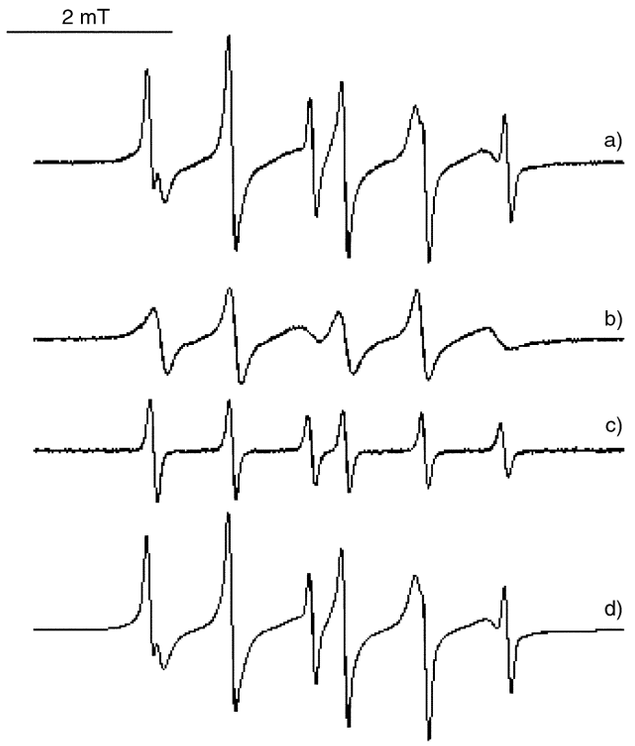

Fig. 1 EPR spectra of TOBER-36 **3** (1.3 mmol L^{-1}) recorded at 35 °C in the presence of a) pH 7.4 HEPES buffer, 150 mmol L^{-1} NaCl and DMPC liposomes (50 g L^{-1}), b) pH 7.4 HEPES buffer, 150 mmol L^{-1} NaCl, DMPC liposomes (50 g L^{-1}) and CROX (50 mmol L^{-1}), c) pH 7.4 HEPES buffer, 150 mmol L^{-1} NaCl, DMPC liposomes (50 g L^{-1}) and ethanol (1 : 1, vol/vol); d) simulation of a) obtained by considering two paramagnetic species, the first one (9%) being **3** in the aqueous phase ($a_N = 1.52$ mT and $a_P = 3.64$ mT, $L_{W-1} = L_{W0} = L_{W+1} = 0.05$ mT), the second one (91%) being **3** in liposomes ($a_N = 1.43$ mT and $a_P = 3.49$ mT, $L_{W-1} = 0.38$ mT, $L_{W0} = 0.17$ mT and $L_{W+1} = 0.21$ mT). The EPR instrument settings were as follows: microwave power, 10 mW; modulation amplitude, 0.1 mT; time constant, 81.92 ms; scan width, 11 mT; scan time, 336 s, receiver gain, 5×10^4.

location of the radicals considered in heterogeneous media. However, significant differences were observed in the behaviour of these radicals in the presence of micelles or of liposomes. All these compounds were found more or less located in the phospholipid bilayer. Although we have no information about the penetration of **1**–**4** into the internal aqueous cavity of the liposomes, these results are encouraging and it is likely that these radicals could present interesting *in vivo* applications. This hypothesis is now to be confirmed by using **1**–**4** directly in the presence of cells.

Experimental

Stable β-phosphorylated aminoxyl radicals **1**–**4** were synthesised and purified in our laboratory as previously described.[7] DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphatidyl-choline) has been purchased from Avanti Polar-Lipids Inc. (Birmingham, AL) and stored at −20 °C. Potassium tris(oxalato) chromate $K_3Cr(C_2O_4)_3 \cdot 3H_2O$ (CROX) and HEPES buffer solutions were obtained from Sigma Chemical Co. All the chemicals were used without further purification. Organic solvents were of the highest grade of purity commercially available.

Unilamellar DMPC liposomes were prepared following a careful procedure indicated hereafter. 75 mg of DMPC were first dissolved in 2 mL methanol–chloroform (1 : 2; vol/vol). DMPC was then deposited on the sides of a round-bottom flask by slow removal of the organic solvent under reduced pressure (380 mmHg) on a rotary evaporator. The resulting dried DMPC film was then hydrated under a nitrogen stream

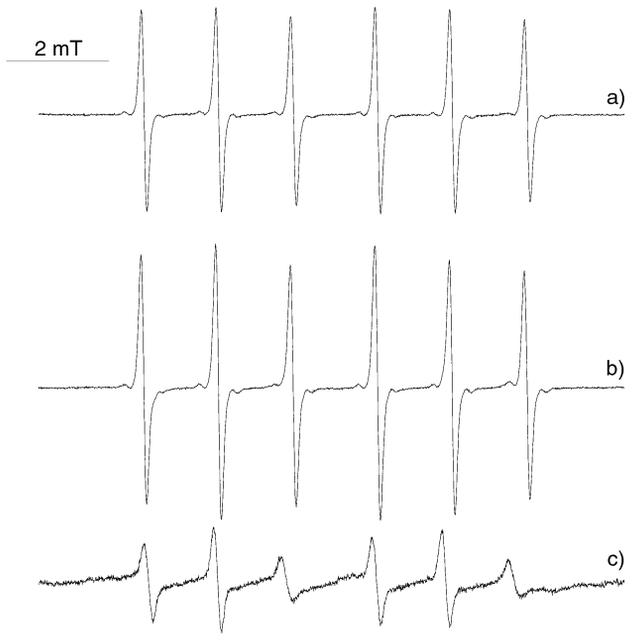

Fig. 2 EPR spectra of TOMER-Et **1** (1.6 mmol L^{-1}) recorded at 35 °C in the presence of a) pH 7.4 HEPES buffer and 150 mmol L^{-1} NaCl, b) pH 7.4 HEPES buffer, 150 mmol L^{-1} NaCl and DMPC liposomes (50 g L^{-1}), c) pH 7.4 HEPES buffer, 150 mmol L^{-1} NaCl, DMPC liposomes (50 g L^{-1}) and CROX (50 mmol L^{-1}). The EPR instrument settings were as follows: a) microwave power, 10 mW; modulation amplitude, 0.1 mT; time constant, 20.48 ms; scan width, 9 mT; scan time, 168 s, receiver gain, 16×10^2; b) microwave power, 10 mW; modulation amplitude, 0.1 mT; time constant, 81.92 ms; scan width, 12 mT; scan time, 168 s, receiver gain, 16×10^2; c) microwave power, 10 mW; modulation amplitude, 0.1 mT; time constant, 81.92 ms; scan width, 12 mT; scan time, 168 s, receiver gain, 64×10^2.

with 1.784 mL of a 150 mmol L^{-1} NaCl solution in pH 7.4 HEPES buffer, and the suspension was vigorously stirred at 30 °C during 15 min with a Vortex mixer. Large multilamellar vesicles were thus produced. The lipid dispersion obtained was frozen in liquid nitrogen and thawed in a 40 °C water bath five times. Extrusion of the frozen and thawed preparations through two stacked polycarbonate filters of 100 nm pore size was twice performed at 30 °C employing a 20 bar nitrogen pressure. The filter device used was a 100 mL Thermobarrel Extruder purchased from Lipex Biomembranes Inc. (Vancouver, B.C.). The aqueous liposome suspension was then filtered using a hand-held syringe fitted with a filter of 450 nm pore size, deoxygenated by argon bubbling and stored at the most 24 hours at 4 °C before use. By this technique, homogeneous unilamellar vesicles with a mean diameter of approximately 90 nm were obtained.

Samples containing unilamellar liposomes (50 mg mL^{-1} DMPC) and one of the stable β-phosphorylated aminoxyl radicals **1**–**4** (concentrations in the range 0.5×10^{-3}–1.6×10^{-3} mol L^{-1}) were prepared in pH 7.4 HEPES buffer containing 150 mmol L^{-1} NaCl. The mixture was then vigorously stirred at 40 °C during 20 min. In some experiments, CROX (final concentration 50 mmol L^{-1}) or ethanol (50% by volume) were added to the medium after the incubation. Then, 20 μL of the final mixture were transferred into a capillary tube for EPR measurements.

EPR spectra were recorded at 35 °C using a computer-controlled Bruker EMX spectrometer operating at the X band with 100 kHz modulation frequency. The following conditions were used: non-saturating microwave power, 10 mW; modulation amplitude, 0.1 mT; receiver gain, from 1.6×10^3 to 50×10^3; time constant, from 1.28 ms to 82 ms; scan time, from 168 s to 672 s; scan width, from 9 to 12 mT. EPR simulations were performed using a software elaborated by Rockenbauer and Korecz.[9]

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