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Original Contribution

Determining radical penetration into membranes using ESR splitting constants

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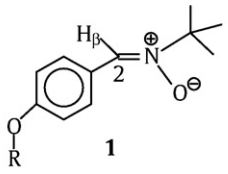
ABSTRACT

Determination of the depth of radical penetration into a lipid membrane is critical to the understanding of the role membranes play in radical attack. We have previously studied radical penetration into lipid bilayers using novel lipophilic spin traps and a combination of NMR and ESR techniques. We now focus on erythrocyte ghost (EG) membranes. Based on a correlation between ESR β-H splitting constants ($a_{\beta\text{-H}}$) and solvent polarity, we have been able to locate stable radicals such as doxyls 2–4 and spin adducts 6–8 intercalated within liposomal bilayers and EG membranes. As a rule, the more lipophilic a spin adduct, the deeper it is found in the bilayer; however, the depth of penetration also depends on the steric bulk of the intercalant and whether intercalation is effected by sonication or diffusion, with the former more energetic and more effective. Compared to simple liposomes, the head group region of the red blood cell membrane is more rigid and lipophilic because of the presence of cholesterol. Hence, the biomembrane head group filters out possible intercalants that are not sufficiently lipophilic. Steric bulk plays less of a role in the EG system, perhaps because the cholesterol introduces a greater element of disorder, attenuating the role played by lipid–lipid interactions.

Introduction

Oxy-radicals, formed in the metabolism of molecular oxygen, play a pivotal role in the "breath of life" (Genesis 2:7). Ironically, though, they also serve as mediators in a host of pathological processes that pose a constant threat to aerobic species [1]. Over the past decade and a half, we have studied the ability of different radicals to penetrate the liposomal phospholipid bilayer, which is a good model for biological membranes. Understanding the reactivity of radicals within liposomal bilayers requires a determination of the depth of radical penetration into the lipid membrane.

In a previous paper [2], we studied the radical penetration of lipid bilayers using a series of novel PBN analogs 1a–i (Eq. (1); PBN is 1j). These homologous spin traps have the same active radical trapping site, the nitronyl carbon (C-2), but differ in their lipophilicity.



1 (1)

a: R= methyl **b**: R= ethyl **c**: R= propyl **d**: R= butyl **e**: R= pentyl **f**: R= hexyl **g**: R= heptyl **h**: R= octyl **i**: R= decyl **j**: OR= H

The intercalation depth of the nitronyl carbon in each of these alkoxy-PBN homologs within the liposomes was determined via a previously reported NMR technique [3–11]. The latter correlates the chemical shifts of selected "reporter" carbons—in the present case C-2, and the micropolarity (measured in Reichardt $E_T(30)$ polarity units [12,13]) experienced by these carbons. We also note that the amphiphilic lipid bilayer contains a polarity gradient [14]—ranging from water [$E_T(30) = 63$ kcal/mol] at the lipid–water interface, down to hexane [$E_T(30) = 31$ kcal/mol] deep within the long-chain fatty acid lipid slab [15]. Hence, the chemical shift data afford qualitative

Abbreviations: EG, erythrocyte ghost; ESR (EPR), electron spin (paramagnetic) resonance; NMR, nuclear magnetic resonance; PBN, N-tert-butyl-α-phenylnitrone; PBS, phosphate buffer solution; PC, phosphocholine; RBC, red blood cell; SA, stearic acids; SLPC, spin-labeled phosphatidylcholines.

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information as to the depth and orientation of the various carbons within the bilayer intercalated compounds.

In our previous work [2], hydroxyl and various α-hydroxyalkyl radicals [$\cdot CH_2OH$, $\cdot CH(CH_3)OH$, and $\cdot C(CH_3)_2OH$] were generated in the extra-liposomal aqueous phase and the lowest depth at which a radical could be spin trapped was determined. The ESR data indicate that these radicals can migrate from the aqueous phase into the DMPC lipid bilayer, penetrating past the phosphatidylcholine head groups ($E_T(30) = 63$ kcal/mol) and past the glycerol esters ($E_T(30) = 52$ kcal/mol) down to an $E_T(30)$ polarity of at least 44 kcal/mol. Other work has shown that this $E_T(30)$ value corresponds to the upper portion of the lipid slab [10]. En passant, we also noted that below an $E_T(30)$ value of 50, the mobility within the bilayer drops drastically as we move toward the lipid slab, and ESR spectra of radicals trapped at these lower polarities show loss of definition and give broad anisotropic ESR signals which acquire a wave-like form.

Since DMPC liposomes do not fully mimic real biological membranes—with the latter's intricate system of special functionalized proteins and sugars—we decided to study the penetration of radicals into more biological systems. To this end we turned our efforts toward erythrocyte or red blood cell (RBC) ghosts.

Materials and methods

General

ESR (EPR) spectra were recorded on a Bruker EPR 1000.d X-band spectrometer at 25 ± 1 °C in a 200 μL flat cell. The ESR measurement conditions were as follows: frequency, 9.67 GHz; power, 20 mW; scan width, 70 G; modulation amplitude, 0.8 G; resolution, 1024; receiver gain, 1×10^4; conversion time, 164 ms; time constant, 655 ms; number of scans, 1 in the case of liposomes and 4 in the case of RBC membranes. Simulation of the recorded spectra was performed using an algorithm provided by the WINSIM program, which is available from the NIEHS-NIH web site (http://tinyurl.com/qpwa2y). We should note that the ESR studies were run at 25 °C. A study carried out in our lab indicated that there is no significant temperature effect on the location of intercalants within the DMPC liposomal bilayer in the 25–40 °C range.

The NMR spectra were recorded on a Bruker AM 300 or Bruker DMX 600 Fourier transform spectrometer. For 1D NMR spectra, we used a QNP probe. NMR spectra were generally taken at 25 ± 1 °C; however, to keep the peaks sharp, NMR spectra of DMPC vesicles were run at 37 ± 1 °C, above the phase transition temperature (T_C) of DMPC (22 °C) [16]. The NMR spectra were generally recorded while locked on the deuterium signals of the respective solvent. The chemical shifts were measured relative to internal tetramethylsilane (TMS), except in the case of aqueous vesicle solutions in which we calibrated the spectrum according to the choline trimethylammonium peak at 54.6 ppm.

Absorption spectra were recorded on a Perkin-Elmer (Norwalk, CT) Lambda-9 UV–visible–near-IR, computer-controlled spectrophotometer and on a Shimadzu (Kyoto, Japan) UV-2501PC UV–vis spectrophotometer.

Other standard equipment included a centrifuge (Sigma 3 K30, Ornate), a vortex (Winn Vortex Genie), a reciprocal water bath shaker (NB-305, N-Biotec Inc.), and a probe sonicator (Soniprep 150 MSE Titanium Probe Ultrasonic Disintegrator, Model MK2, at a 20 KHz output frequency).

Carbon monoxide (CO), potassium oxalate, Tris-HCl, EDTA, HEPES, dimyristoyl phosphatidylcholine (DMPC), and the deuterated solvents were obtained from Sigma-Aldrich Chemical Company. 1-Palmitoyl-2-stearoyl-(10-doxyl)-sn-glycero-3-phosphocholine (2a), 1-palmitoyl-2-stearoyl-(12-doxyl)-sn-glycero-3-phosphocholine (2b), 1-palmitoyl-2-stearoyl-(16-doxyl)-sn-glycero-3-phosphocholine (2c), 1,2-dipalmitoyl-sn-glycero-3-phospho-TEMPO-choline (TEMPO-PC, 3), 5-doxyl stearic acid (5-SA), and 16-doxyl stearic acid (16-SA) were purchased from Avanti Polar Lipids, Alabaster, Germany. All PBN derivatives (1a–i) were freshly synthesized according to Gamliel et al. [2]. Doubly purified water (via Millipore columns) containing 1.7 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, and 2.7 mM KCl and 137 mM NaCl were used in the preparation of a phosphate buffer solution, pH 7.4; the latter was utilized, in turn, to prepare all aqueous solutions, unless otherwise indicated. The RBC lysis medium was prepared using 10 mM Tris-HCl and 0.2 mM EDTA with doubly purified water to give a pH of 7.2. KCl buffer was prepared with 150 mM KCl, 10 mM HEPES and 0.5 mM EDTA in doubly purified water to give a pH of 7.4.

General procedure for the preparation of DMPC liposomal solutions by sonication or diffusion

The general procedure for the preparation of DMPC liposomal suspensions by sonication for the NMR studies has been previously described [2,6]. In the preparation of liposomes intercalated by diffusion, a solution of empty liposomes was prepared as described previously [7]. The compound to be intercalated (henceforth dubbed the "intercalant") was dissolved in a small amount of ethanol (no more than 2% by volume of the total buffer solution) and added to the empty liposome solution. The latter was shaken for several hours using a Winn Vortex Genie. When noted, samples were washed by first centrifuging the liposomes down to a pellet at 20,000 *g* for 15 min. The supernatant water was removed and clean buffer solution was added. The solution was gently stirred and this process was repeated twice more.

General procedure for ESR measurements

The general procedure for the preparation of DMPC liposomal suspensions and the Fenton System for the ESR studies have been previously described [2]. When creating spin adducts prior to intercalation into membranes or liposomes, the Fenton reagent was added to a solution of the substrate in no more than 300 μL ethanol. The suspension was then added to the membranal or liposomal solution whose preparation has been described [2]. We note that all samples prepared for ESR measurements (unless otherwise stated) were washed—by centrifugation, decantation of supernatant liquid, and resuspension in new buffer—at least thrice before running ESR spectra. Controls were run for each experiment. In particular, when lipophilic spin traps 1 were intercalated into liposomes by sonication, controls verified that only negligible amounts of ROS are detected.

Calibration of the relative spin label solution concentrations

Although each commercial sample had the same initial concentration when purchased, spin labels tend to decompose slowly on standing. Prior to use, we determined the relative concentration of these radical solutions using the following method. Equal volumes of the 6 spin label solutions (2a, 2b, 2c, 3, 4a, and 4b) were diluted in 750 μL of ethanol and ESR spectra were run. This was repeated 3 times for each sample. As expected, all the signals received were triplets—due to the hyperfine interaction of the electron spin with the nuclear spin of ^{14}N. Double integration of the central peak reflects the radical concentration. The relative concentration of each radical was then calculated.

General procedure for the preparation of erythrocyte (RBC) ghost suspensions

Ghosts were prepared as described by Cloherty et al. [17]. Red blood cells were isolated from whole human blood by repeated wash/centrifugation cycles in ice-cold PBS. One volume of whole blood was mixed with 3 vol of PBS and centrifuged at 3000 *g* for 5 min at 4 °C. Serum and the buffy coat were aspirated, and the wash/centrifugation cycle was repeated until the supernatant was clear and a buffy white coat was no longer visible. Usually this cycle was repeated thrice. The washed red blood cells were lysed in 40 vol of lysis medium, incubated on ice for 10 min, and then centrifuged at 20,000 *g* for