Enzymically Generated Electron-excited Species Increase the Membrane Order Parameter without Changing the Rotational Relaxation Time of TMA-DPH

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Abstract. The effect of enzymically generated triplet acetone produced from 60 mmol/l 2-methylpropanal (MPAL) in the presence of 2 μmol/l horseradish peroxidase on the properties of artificial unilamellar liposomes was studied. The concentration of malondialdehyde, membrane lateral pressure (membrane order parameter), lifetime distribution of the excited state of TMA-DPH incorporated into liposomes, and rotational relaxation time of TMA-DPH were determined. The concentration of MDA increased with the MPAL concentration, and the membrane lateral pressure increased linearly with the concentration of MDA. The mean lifetime values changed ($p < 0.01$) as did the limiting anisotropy ($p < 0.005$), the widths of the main peak of the lifetime distribution and the rotational relaxation time remaining unchanged. This indicates that there is no significant difference between the widths and rotational relaxation times in the control and triplet-acetone-treated sample; i.e. peroxidation by MPAL increased the lipid order of the bilayer but the effect of neighboring lipid molecules on the dynamics of the probe was negligible.

Key words: Electron-excited species — Lipid peroxidation — Membrane order parameter — Rotational relaxation time — TMA-DPH — Fluorescence (spectroscopy)

Introduction

Polyunsaturated fatty acids in membrane lipids can be modified and degraded by lipid peroxidation, a branched chain reaction of free radicals (Tappel 1972; Valen-

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zeno 1987). Such a modification of membrane lipids can subsequently influence membrane-bound proteins, resulting in a change of their structural and functional properties (Marin et al. 1992; Ohta et al. 1989). To distinguish between a direct modification of the membrane proteins by peroxidation and effects mediated by lipids is not a simple task, resembling that of defining the molecular mechanism of lipid-mediated effects.

The obvious first step in this direction is to describe the molecular mechanism of peroxidation of membrane lipids without any protein present. Artificial unilamellar liposomes prepared from phosphatidylcholine were used to study the effect of lipid peroxidation on membrane order and on rotational relaxation time, as determined by time-dependent depolarization of fluorescence of TMA-DPH.

Abbreviations: TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid; MDA, malondialdehyde; TMA-DPH, trimethylammonium-1,6-diphenyl-1,3,5-hexatriene; POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene; SOD, superoxide dismutase (EC 1.15.1.1); MPAL, 2-methylpropanal; HRP, horseradish peroxidase (EC 1.11.1.7).

Materials and Methods

Preparation of unilamellar liposomes

Unilamellar liposomes were prepared by a probe sonicator (70 mg of L-α-lecithin on 10 ml 30 mmol/l Tris-HCl buffer, pH 7.2) under the inert atmosphere of nitrogen (Wilschut 1982). After sonication, metal particles from the probe and large multilamellar liposomes were removed by ultracentrifugation for 1 h at 100,000 g.

Generation of triplet acetone and induction of lipid peroxidation

Triplet acetone was generated by oxidation of different concentration of 2-methylpropanal in the presence of 2 μmol/l horseradish peroxidase (HRP) in 20 mmol/l phosphate buffer at pH 7.2 for 60 min at 25 °C (Cilento 1984). The total volume was 1 ml, concentration of lecithin liposomes was 0.2 mmol/l.

Induction of lipid peroxidation by Fe^{2+} plus ascorbic acid

Liposomes (0.2 mmol/l lecithin) were incubated for different periods of time with 0.1 mmol/l $FeSO_4$ and 0.4 mmol/l ascorbic acid at 25 °C. The reaction was started by the addition of ascorbic acid and stopped by the addition of 5 mmol/l EDTA.

Detection of malondialdehyde (MDA)

One ml of a mixture of 0.2 mmol/l lecithin liposomes, 25 mmol/l TBA, 0.9 mol/l TCA and 50 mmol/l HCl in a test-tube was heated to 95 °C for 60 min and then vortexed for 15 min with 2 ml of n-butanol. The concentration of MDA was calculated from the absorption ($\epsilon = 1.56 \times 10^5$ mol.l^{-1}.cm^{-1}) of the organic layer at 532 nm with n-butanol as absorbance blank (Ohkawa et al. 1979).

Absorption and steady-state fluorescence measurements

Absorption measurements were done on a Uvikon 810 spectrophotometer. Steady-state fluorescence data were taken either on a Perkin-Elmer LS-5 or a Perkin-Elmer 7300 fluorometer, both with monochromators, and in quartz cuvettes with reflecting walls (Amler et al. 1992). All measurements were done at 25 °C. TMA-DPH was dissolved in acetone. Stock solution was 1 mmol/l, the final concentration in the cuvette was 3 μmol/l. Excitation and emission wavelengths were 365 nm and 430 nm, respectively. Two Glan-Thompson polarizers were used for detection of steady-state anisotropy values. Membrane lateral pressure was calculated from the steady-state anisotropy values according to Fulford and Peel (1980).

Dynamic fluorescence measurements

Frequency-domain measurements were done to determine the lifetimes of the excited states and rotational correlation times using an ISS K2 Multifrequency Cross-Correlation Phase and Modulation Fluorometer (ISS, Urbana, USA). Phase and modulation measurements were done in the range from 5 to 100 MHz with a xenon lamp as the source of excitation light and a monochromator. Fluorescence emission from the samples was observed through Schott filters. Cross-correlation frequency was 80 Hz, integration time 30 s and maximum errors were 0.2 for phase and 0.005 for modulation, respectively. The frequency response was observed for 10 different frequencies equidistant on a logaritmic scale over the frequency range from 5 to 100 MHz. A solution of POPOP in methanol was used as a reference sample. Corrections for background fluorescence and/or light scattering were taken as frequency-domain measurements with a nonlabeled sample as control and then correcting the data in the manner described by Lakowicz et al. (1987). The experimental data were fitted using a nonlinear least-squares routine analysis for multiexponential decays or lifetime distributions.

Results

1. Mechanism of initiation of lipid peroxidation by triplet acetone

The transfer of excitation energy from triplet acetone to dissolved oxygen leads to generation of singlet oxygen and/or superoxide radicals which initiate lipid peroxidation (Foote 1991). In fact, lipid peroxidation induced as described above can be the consequence of either the presence of singlet oxygen or the appearance of superoxide radicals. Lipid peroxidation was brought about by triplet acetone, using methods recently developed. Triplet acetone was generated by oxidation of 2-methylpropanal with horseradish peroxidase in the presence of deuterium oxide or superoxide dismutase (Cilento 1984). A compound of dioxetane type is presumed as the oxidation intermediate which is decomposed into excited triplet acetone and formic acid. The transfer of triplet energy to a suitable acceptor can be utilized for inducing a photochemical reaction, often called "photochemistry without light", which takes place both in plants and animals (Cilento 1988).

Production of malondialdehyde, the dominant secondary product of lipid peroxidation, is a consequence of degradation of polyunsaturated fatty acids (Gardner

Table 1. Induction of lipid peroxidation by triplet acetone in the presence of deuterium oxide and superoxide dismutase

Addition	Concentration of MDA (nmol MDA/mg of phospholipids)
None	7 15 ± 0 20
Deuterium oxide (60%)	9 79 ± 0 25
SOD (150 units)	7 10 ± 0 15

Incubation was done in 20 mmol/l phosphate buffer, 60 mmol/l MPAL, 2 μmol/l HRP, pH 7 2, with 0 2 mmol/l lecithin liposomes for 60 min at 25 °C Concentration of MDA was used as a measure of lipid peroxidation The average values and standard errors are averages of four independent experiments

1989) Thus, the concentration of MDA can and was used here as a measure of lipid peroxidation (Ohkawa et al 1979)

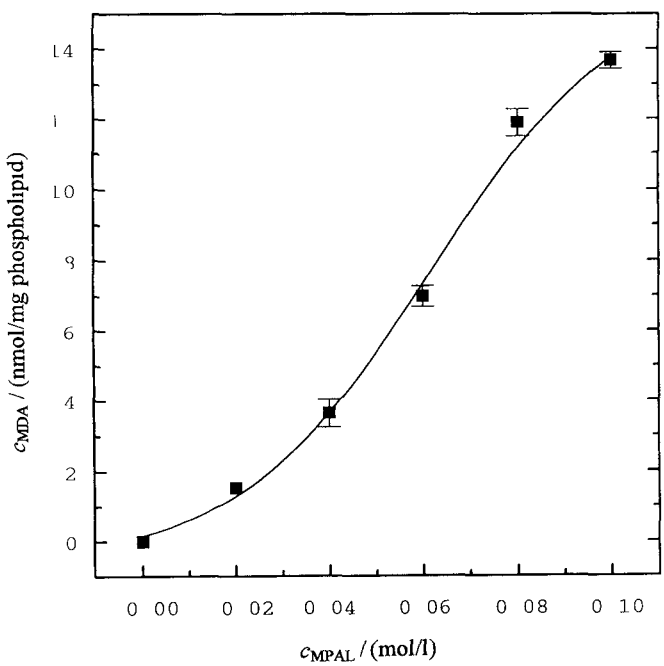

Figure 1. Induction of lipid peroxidation by triplet acetone Peroxidation in unilamellar lecithin (0 2 mmol/l) liposomes was generated by incubation of different concentrations of 2-methylpropanal (MPAL) in the presence of horseradish peroxidase (HRP) Concentration of MDA was used as a measure of lipid peroxidation The experimental points are averages of five independent measurements

Singlet oxygen did develop (Table 1) as suggested by the higher concentration of MDA in the presence of deuterium oxide (singlet oxygen has a longer life-time in the presence of deuterium oxide). Superoxide dismutase remained without any effect which excludes the possibility of superoxide radical generation.

2. Effect of MDA on steady-state anisotropy of TMA-DPH

Dependence of peroxidation on MPAL concentration

The extent of peroxidation of liposomes was determined at different concentrations of MPAL. Five independent measurements were done at each concentration and the averages and standard deviations are plotted in Fig. 1. At the highest concentration of MPAL tested (0.1 mol/l), peroxidation was close to saturation, indicating that the lipids were totally peroxidized. Higher concentrations of MPAL were avoided since they might affect the activity of horseradish peroxidase.

MDA was also generated in the presence of Fe^{2+} and ascorbic acid. Incubation times of liposomes ranged from 0 to 200 min when the limiting value (total peroxidation) was reached (see Fig. 2).

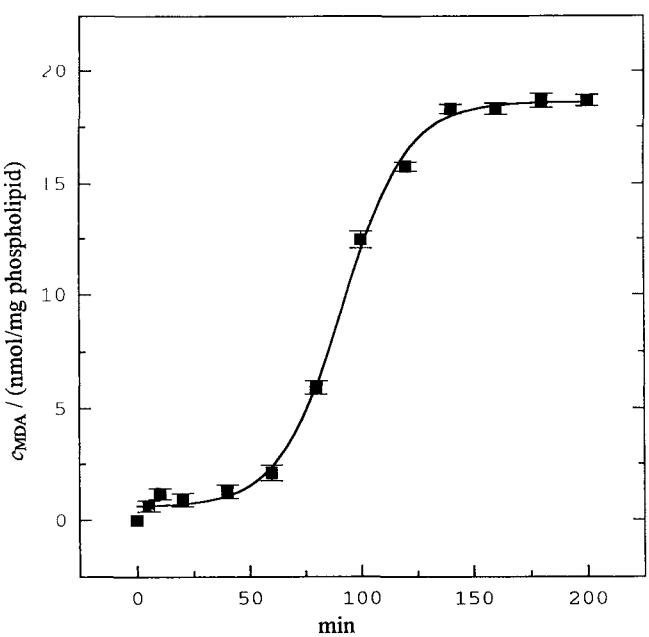

Figure 2. Induction of lipid peroxidation by Fe^{2+} and ascorbic acid. Peroxidation in unilamellar lecithin (0.2 mmol/l) liposomes was induced by incubation for different intervals with ascorbic acid and $FeSO_4$. Concentration of MDA was used as a measure of lipid peroxidation. The experimental points are averages of four independent determinations.

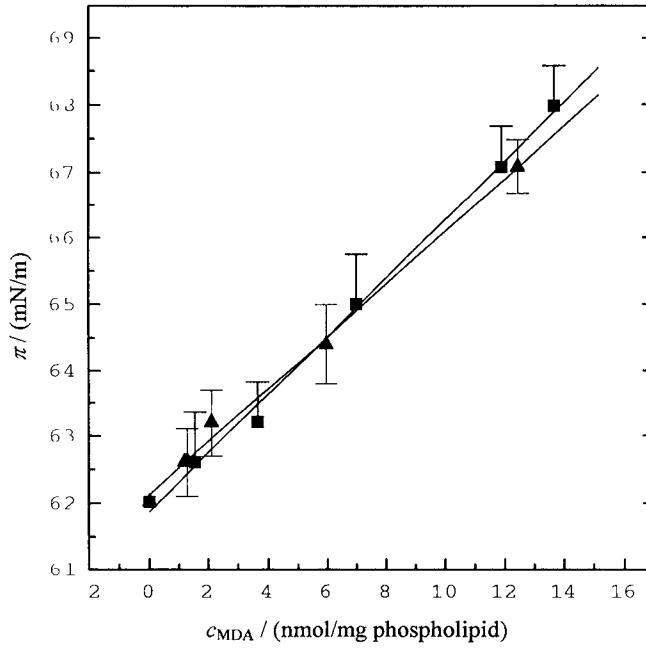

Figure 3. Dependence of membrane lateral pressure on peroxidation. Membrane lateral pressure π (in mN/m) was calculated from steady-state anisotropy of TMA-DPH (3 μmol/l) in unilamellar lecithin liposomes. Changes of membrane lateral pressure were induced by the system MPAL plus HRP (squares) or of ascorbic acid plus $FeSO_4$ (triangles). Mean values and experimental errors are averages from five independent experiments.

Influence of peroxidation on membrane lateral pressure

The effect of peroxidation on the membrane lateral pressure was tested and peroxidation by triplet acetone was compared with the classical system of Fe^{2+} plus ascorbic acid. Steady-state fluorescence anisotropy of TMA-DPH was used to determine the membrane lateral pressure according to Fulford and Peel (1980). TMA-DPH is a fluorescent probe which is distributed at the membrane-water interface and thus yields information on the effects in that region. Membrane lateral pressure was plotted (Fig. 3) as a function of nmol MDA per mg phospholipid where the correlation with MPAL concentration or the time of incubation with Fe^{2+} and ascorbic acid as described in Figures 1 and 2 were employed. In both systems the membrane lateral pressure increased linearly with MDA concentration. In addition, the slopes of the linear dependence were indistinguishable within the error of measurement. This indicates that both systems influenced the lipid bilayer in a similar way.

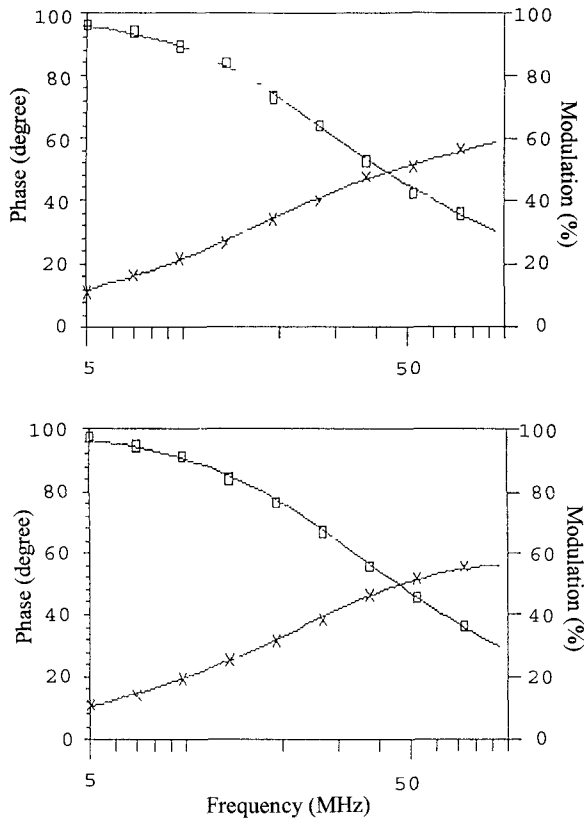

Figure 4. Frequency response of TMA-DPH in unilamelar liposomes. Frequency response (squares for phase, crosses for modulation) was measured over the frequency range of 5–100 MHz in control unilamellar liposomes (top) and after peroxidation (bottom). Thè curves stand for theoretical values of the best fit (see Fig. 5). Lipid concentration was 1 mg/ml, concentration of TMA-DPH was 5 μmol/l. Temperature of measurement was 25 °C.

3. Effect of triplet acetone on the average lifetime of the excited state and the rotational relaxation time of TMA-DPH

To establish whether the observed steady-state changes of TMA-DPH (see Fig. 3) were due to changes of limiting anisotropy or to changes of the rotational relaxation time of the probe, a dynamic experiment was done. Unilamellar phosphatidylcholine liposomes were tested with MPAL sufficient to reach the maximum of the steady-state anisotropy value, i.e. incubated with 0.1 mol/l MPAL for 60 min in 50 mmol/l Tris-HCl at pH 7.4. Such a system with final lipid concentration of 1 mg per ml was subsequently labeled with 5 μmol/l TMA-DPH and both fluorescence

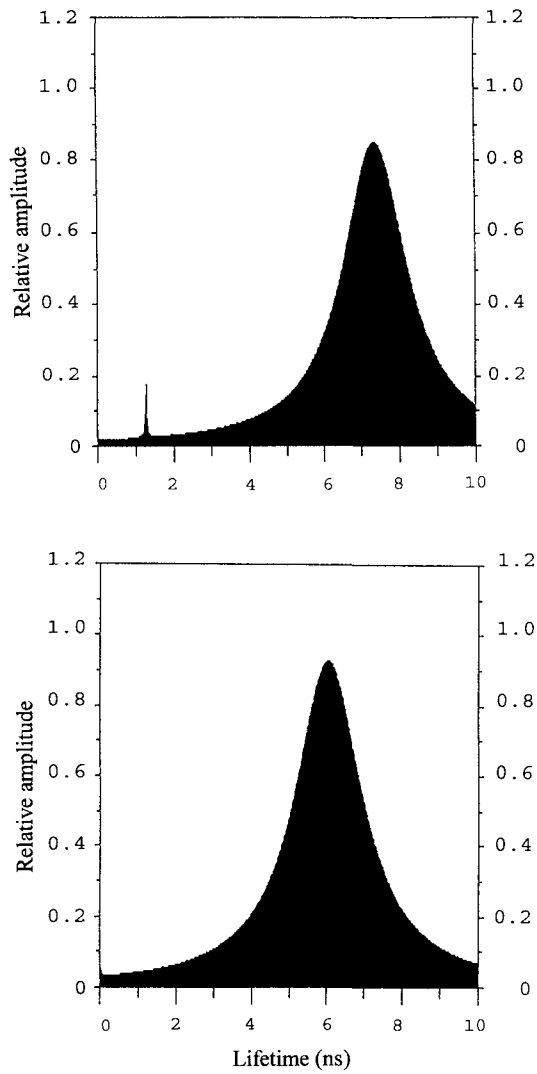

Figure 5. TMA-DPH lifetime distribution before and after peroxidation. Two-component Lorentzian distributions appeared as the best fit for both the frequency responses with the major components centered at 7.29 ns (control) and 6.04 ns (sample), with widths of 2.25 ns (control) and 2.19 ns (sample) and goodness of fit $\chi^2_R = 7.6$ (control) and $\chi^2_R = 7.9$ (sample). A minor fraction centered at 1.24 ns appeared also in the control sample. This component almost disappeared after peroxidation.

intensity and anisotropy decay were followed by the frequency-domain method over the range of 5–100 MHz (Fig. 4).

Determination of the lifetimes of the excited state of TMA-DPH incorporated into treated and untreated liposomes showed a clear difference (Fig. 5). However, there was no difference in the lifetimes between the liposome preparation alone and that after adding Tris buffer without the reagents. Data analysis revealed that the best fit of the data for the control was a Lorentzian lifetime distribution with two lifetime values centered at 7.29 ns (fraction $f = 0.86$) and 1.24 ns with the widths of distribution (w) of the former 2.25 ns and 0.05 ns of the latter (see Fig. 5). While the two-component Lorentzian distribution appeared as the best fit for the measured data also in the sample with generated triplet acetone, the maxima were shifted. The main (longer) lifetime of the excited state decreased to 6.04 ns with a width of 2.19 ns; the minor component was 0.05 ns with a very narrow width of 0.05 ns (the minimum width allowed by the software used for the analysis). The appearance of the minor component is quite common in samples labeled with DPH or its derivatives. The origin of this minor, short-lived component, even if not fully understood, is usually ascribed to some kind of photo-effect. Processes taking place in the lipid bilayer are, however, reflected by the major component.

Table 2. Limiting anisotropy and rotational relaxation time of TMA-DPH

	Limiting anisotropy	Relaxation time (ns)
Control	0.257 ± 0.006	1.6 ± 0.2
Treated	$0\ 285 \pm 0.008$	1.6 ± 0.3

The decay data were calculated as an average of three determinations.

To understand better the effect of triplet acetone on lipid bilayers, we also measured the differential tangent in both the control and treated samples. The limiting anisotropy and rotational relaxation time were calculated using the software available in the Institute of Biochemistry, University of Ancona, Italy. The results are summarized in Table 2. Clearly, while the mean lifetime values differed ($p < 0.01$) similarly as did the limiting anisotropy ($p < 0.005$), the widths of the main peak of the lifetime distribution and the rotational relaxation time remained unchanged. This means that there is no significant difference between the widths and rotational relaxation times in the control and triplet-acetone-treated sample; i.e. peroxidation by triplet acetone increased the lipid order of the bilayer but interaction of the fluorescent probe with neighboring lipid molecules remained unchanged.

Discussion

It is shown here that triplet acetone generated by oxidation of 2-methylpropanal under catalysis of horseradish peroxidase triggers lipid peroxidation. Appearance of malondialdehyde in the presence of deuterium oxide (while superoxide dismutase remained without any effect) indicates the mechanism of the initiation of lipid peroxidation as excitation energy transfer from triplet acetone to oxygen which leads to production of singlet oxygen.

Notably, the effectivity of peroxidation of singlet oxygen and of hydroxyl radicals (generated by the system of ascorbic acid plus Fe^{2+}) was similar. In other words, enzymically generated electron-excited species could be an alternative source of reactive forms of oxygen and, thus, can contribute to developing oxidative stress in living organisms. Most importantly, enzymically generated electron-excited species can induce a "photochemical-like" peroxidation in the dark due to light-insensitive reactions.

As to the molecular mechanism of the singlet oxygen effect on the lipid bilayer, it can be concluded that

(a) "microviscosity" derived from the rotational relaxation time of DPH derivatives remained unchanged within the experimental error after generation of singlet oxygen in the lipid bilayer; this means that the observed steady-state anisotropy changes reflected changes of limiting anisotropy (membrane order) only;

(b) singlet oxygen stabilized the membrane structure which resembles the effect of vanadyl ions described earlier (Amler et al. 1986); these changes in the structural arangement of the lipid compartment at the membrane can consequently influence the functional properties of membrane bound proteins as is well known in cases of oxidative stress.

Acknowledgements. This study was supported by grants No. 203/93/2467 and No. 204/93/0703 from the Grant Agency of the Czech Republic.

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Final version accepted October 5, 1995