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Permeation of superoxide anion through the bilayer of vesicles of a synthetic amphiphile

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Large unilamellar vesicles, prepared with dioctadecyldimethylammonium chloride, entrap nitroblue tetrazolium. Addition of solid KO_2, or production of superoxide anion by riboflavin photolysis, to nitroblue tetrazolium-containing dioctadecyldimethylammonium vesicles results in the formation of monoformazan above the phase-transition temperature of the bilayer. Below the phase-transition temperature the yield of monoformazan is negligible. These results demonstrate that superoxide anion permeates vesicles above the phase-transition temperature of the bilayer.

Introduction

Oxygen toxicity is one of the prices paid for the acquisition of aerobiosis during evolution. Active oxygen species (AOS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$), singlet oxygen and chemical reactive species derived from AOS's chemical reactions, may damage a variety of essential cellular components [1]. O_2^- dismutation and the resulting production of H_2O_2 and $OH^\cdot$ are early steps in the cellular AOS-driven reactions [1]. Nitric oxide, through reaction with O_2^-, can also mediate $OH^\cdot$ production in biological systems [2]. Since $OH^\cdot$ reactions are diffusion controlled it is unlikely that this radical reacts far from the production site [1]. Thus, diffusion of O_2^- may be one of the determining factors of cellular targeting of $OH^\cdot$ reactions. Membranes are the main intracellular diffusional barriers and, despite suggestions that O_2^- may permeate, transmembrane O_2^- diffusion is not generally accepted [3–9].

The use of biological membranes as a system for the study of O_2^- diffusion creates experimental problems. O_2^- may chemically react with membrane components and, consequently, the permeability of the membrane may be affected by O_2^- reacting with the experimental system [10,11]. It is convenient, therefore, to use a simpler and chemically unreactive system to investigate if O_2^- can indeed permeate a bilayer.

In this communication we demonstrate, using a model system composed of vesicles prepared with a synthetic amphiphile, that O_2^- permeates a membrane above the phase-transition temperature (T_c) of the bilayer.

Materials and Methods

Riboflavin (Fluka), EDTA (Reagen Quimibras), Brij-35 (Calbiochem) and KO_2 (E. Merck) were used as received. Nitrobluetetrazolium (NBT, Sigma) was recrystallized three times from cold ethyl acetate/methanol [12].

Hexadecyltrimethylammonium chloride (HTAC) was prepared from HTA bromide (E. Merck) by ion-exchange in Dowex 21K-Cl (Serva). Commercial grade dioctadecyldimethylammonium chloride (DODAC) (Herga) was dried under vacuum and extracted with ethyl ether. The solid fraction was then recrystallized 6-times from acetone and dried under vacuum [13]. The resulting amphiphile had a chain composition of 75% C_{18}/C_{18}, 23% C_{18}/C_{16} and 2% C_{16}/C_{16} as determined by gas chromatography and mass spectrometry [13]. All other reagents were analytical grade. Deionized, doubly-distilled (glass) water was used throughout.

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Preparation of stock solutions of KO_2

Stock solutions of KO_2 were prepared as follows: 5 mg of KO_2 were weighted (dry box) into tubes that were then tightly stoppered and, immediately before use, 1.0 ml of ice-cold NaOH 0.050 M was added. Thirty seconds after the addition of NaOH, a 50-μl aliquot of the stock solution was added to the reaction mixtures. The remaining stock solution of KO_2 was discarded. Careful standardization of this procedure yields solutions in which the initial concentration of O_2^- is approx. $(1-2) \cdot 10^{-5}$ M, based on a molar extinction coefficient of $2.0 \cdot 10^3$ $M^{-1}cm^{-1}$ at 250 nm [14].

Superoxide anion generation by riboflavin photolysis

The photolysis system was built to permit irradiation of 2.0-ml samples inside quartz cuvettes of 1.0 cm optical path. The cuvettes were placed inside a thermostatted cell (0.98 cm^2 slit) over a magnetic stirrer. The distance between the lamp (Osram halogen 1, 12 V/55 W) and the cuvette was 8.0 cm. A cylindrical lens provided a clear image of the lamp filament at this distance. A Corning filter (CS-3-72, 2.93 mm) was used to cut off light below 420 nm. After irradiation absorption spectra were recorded in a Beckman DU-7 spectrophotometer, using the (corresponding) unphotolyzed samples as reference. Relative fluorescence intensities were used to estimate the decrease in riboflavin concentration (Perkin-Elmer LS-5 spectrofluorimeter). The first-order rate constants of NBT reduction by steady state concentrations of O_2^- in the photolysis experiments (k_ψ) were estimated from the variation in the absorbance of monoformazan (see Results) at 530 nm with irradiation time. The data fitted a first-order process and k_ψ was calculated using a non-linear least squares program.

Vesicle preparation and permeability assay

Large unilamellar vesicles containing NBT were prepared by injection of a 0.020 M chloroformic solution of DODAC [15] in borate buffer (0.01 or 0.05 M, pH 9.5 or 8.2, respectively) containing $2 \cdot 10^{-3}$ M NBT and then annealed by incubation for 1 h at 45°C and slowly cooled. The non-entrapped (external) NBT was removed by gel filtration using Sephadex G-25. Vesicles were used within 12 h of the preparation. Sephadex G-25 chromatography was repeated and NBT contents determined to measure NBT leak from the internal vesicle compartment. At room temperature the vesicle preparations were stable and did not leak NBT for three days. NBT content of the vesicles was measured spectrophotometrically at 260 nm (molar extinction coefficient $6.4 \cdot 10^4$ $M^{-1}cm^{-1}$ [16]) after vesicle dissolution by heating at 50°C for 2 min with 0.1 M HTAC. DODAC concentration was measured as described [17]. Permeability of O_2^- was determined by KO_2 addition to a suspension of vesicles containing NBT (final concentration $6.2 \cdot 10^{-6}$ M) or by photolysis of riboflavin with methionine ($2 \cdot 10^{-3}$ M) instead of EDTA as the electron donor.

All data are the result of (at least) two separate experiments done in duplicate or triplicate (in the case of KO_2 addition).

Results and Discussion

Quantitative analysis of superoxide anion in the presence of micelles and vesicles

The detection of yields and the measurement of rates of O_2^- permeation relies on analytical methods capable of determining the concentration of superoxide anion in experimental systems that generally contain complex membrane systems. To date, there is no general analytical method appropriate for all experimental conditions. The reactions most commonly employed for O_2^- detection include the reduction of cytochrome *c* [18] and several tetrazolium compounds [19–22], as well as the oxidation of epinephrine [23]. The reduction of nitroblue tetrazolium (NBT), under conditions where the only product is the corresponding monoformazan (MF) [19–22], was selected as the most convenient analytical detection method for our purposes. NBT is freely soluble in aqueous solution, exhibits a high extinction coefficient, its rate of reaction with O_2^- is fast and MF deoxidizes spontaneously much slower than the corresponding cytochrome *c* [24].

The analysis of O_2^- transport across membranes may be further complicated, since interfaces can modify the rates of reaction [25] and, consequently, affect the rate of O_2^- dismutation. We checked for this possibility measuring the effect of micelles on the rate of O_2^- decomposition. The rate constant (k_d) for spontaneous decay of O_2^- in buffered solutions (pH 9.5), calculated from the decay in absorbance of O_2^- at 250 nm with time, was 0.2 s^{-1}, well within published values [14, 26]. Addition of hexadecyltrimethylammonium chloride (HTAC) micelles or vesicles of dioctadecyldimethylammonium chloride (DODAC) did not affect k_d. Hence, at least in these systems, added micelles or vesicles do not affect the rate of decomposition of the diffusing species.

Addition of KO_2 to an air-equilibrated aqueous buffered solution containing NBT resulted in a product with UV spectrum identical to monoformazan (MF) (Fig. 1) [22]. The formation of MF increased linearly with added KO_2 (Fig. 1).

Photolysis of riboflavin produced O_2^- in NBT-containing solutions yield the same product. Increasing the irradiation time produced an increase in the absorbance of MF and the isosbestic point at 305 nm indicates the formation of only one product (Fig. 2).

In the presence of HTAC micelles or of DODAC vesicles the spectrum of MF is similar to that in aque-

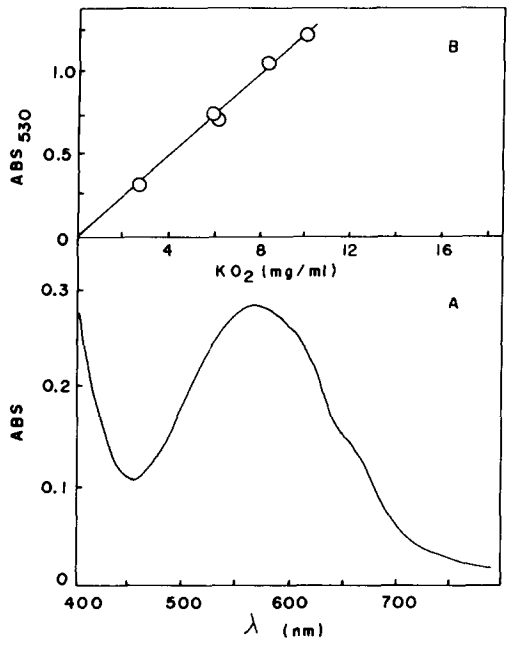

Fig. 1. (A) Visible absorption spectra of monoformazan (MF) produced upon reaction of nitrobluetetrazolium (0.04 M) with potassium superoxide (approx. $1 \cdot 10^{-5}$ M). (B) Effect of KO_2 concentration on the formation of MF at 530 nm.

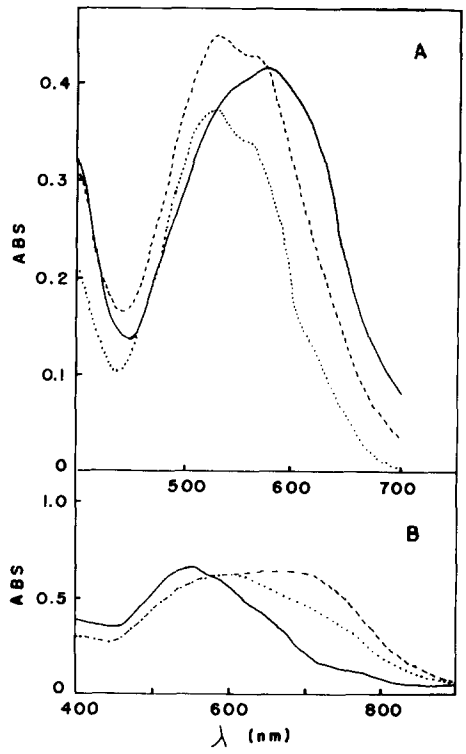

Fig. 3. Solvent and temperature effects on the spectra of monoformazan (MF). (A) Visible absorption spectra of MF in ethanol (· · · · · ·); Brij-35, 0.004 M (— — —) and HTAC 0.004 M. MF was obtained by reduction of NBT with ascorbate (in ethanol) or KO_2, respectively. (B) Spectra of MF obtained after reduction of NBT by KO_2 in a solution containing DODAC ($1 \cdot 10^{-4}$ M) vesicles. Samples were thermostatted at 25°C (———), heated to 45°C (— — —) and cooled to 25°C (· · · · · ·).

ous solutions. In Brij 35 micelles the spectrum of MF is comparable to that in ethanolic solutions (Fig. 3A). The spectra of vesicle-incorporated MF changed with temperature (Fig. 3B), as described for other amphiphile aggregate-incorporated substrates [25].

The rate constant of MF formation (k_ψ), upon O_2^- production by riboflavin photolysis, was not affected by micelles of HTAC (Fig. 4).

The data presented so far show that MF formation adequately measures O_2^- concentration and that this method can be used in the presence of HTAC and Brij micelles as well as DODAC vesicles. Therefore, it is possible to employ this determination method to evalu-

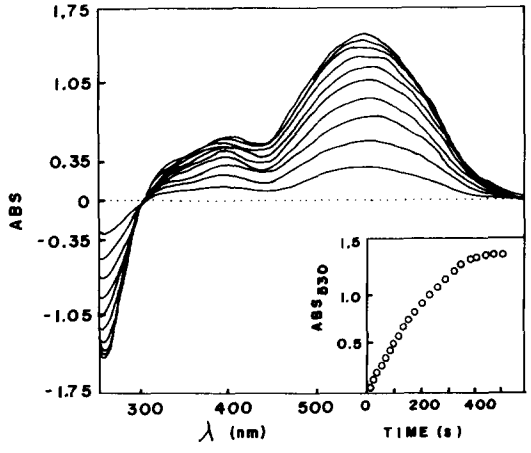

Fig. 2. Production of monoformazan (MF) upon photolysis of riboflavin in the presence of nitrobluetetrazolium (NBT). Samples contained: 0.04 M NBT, $1.4 \cdot 10^{-6}$ M riboflavin, 0.02 M EDTA, 0.004 M HTAC and 0.05 M borate buffer (pH 8.2). The spectra of samples irradiated for increasing periods of 1 min are presented. Inset: A_{530} against irradiation time.

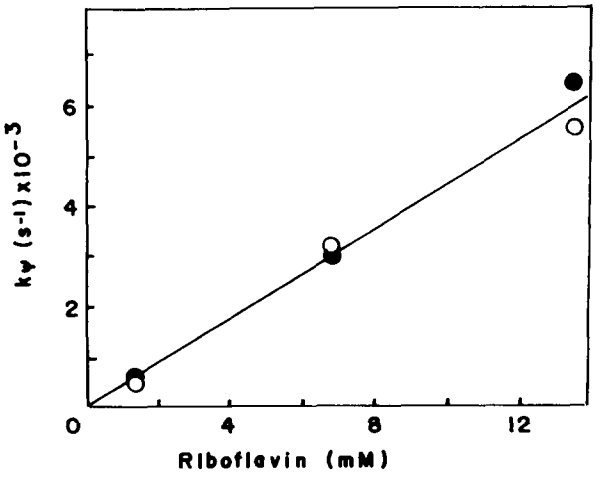

Fig. 4. Effect of riboflavin concentration on the first order rate constant for the formation of monoformazan (MF) in the presence of nitroblue tetrazolium (NBT). Irradiation samples contained 0.02 M EDTA, $4 \cdot 10^{-5}$ M NBT and 0.02 M phosphate buffer pH 7.6 without (○) or with HTAC ($4 \cdot 10^{-3}$ M) (●).

ate O_2^- permeation across the bilayer of DODAC vesicles. This analytical method is not adequate to determine O_2^- concentration in the presence of egg-lecithin vesicles, since NBT decomposes to give a complex product mixture (not shown).

Superoxide ion permeation through bilayers of DODAC vesicles

The phase-transition temperature (T_c) of large DODAC vesicles is 38°C [15] and the O_2^- diffusion experiments were done below (25°C) and above (45°C) the T_c. The photolysis of riboflavin yields steady-state concentrations of O_2^- that are maintained during photolysis. The second-order rate constant for NBT reaction with O_2^- is $6 \cdot 10^4$ $M^{-1}s^{-1}$ [22,26]. Upon addition of KO_2 the initial concentration of O_2^- was approx. 10^{-5} M and in the photolysis experiments NBT concentrations were of the same order of magnitude. Therefore, the reaction of O_2^- with NBT should not be rate limiting in our experimental conditions.

The k_ψ at 45°C was similar to that obtained adding DODAC vesicles or entrapping NBT in DODAC vesicles, strongly suggesting that O_2^- rapidly permeates the bilayer above the T_c (Table I). At 25°C, NBT entrapment in DODAC vesicles decreased the value of k_ψ 3-fold (Table I). Preincubation of the vesicles at 45°C without riboflavin further decreased k_ψ 30-fold (Table I). The values of k_ψ obtained by preincubation of NBT-containing vesicles with (external) riboflavin at 45°C, before photolysis at 25°C, were higher than water (Table I).

Below the T_c large DODAC vesicles are impermeable to most anions, with the exception of OH^- that permeates slowly into annealed DODAC vesicles [15,27]. Chain interdigitation in synthetic amphiphile vesicles can be extensive and preincubation above the T_c yields a more ordered bilayer [28]. The results presented in Table I show that permeation of O_2^- through DODAC bilayers below the T_c is slow and may be ascribed to bilayer defects, since it is essentially suppressed by further annealing. The (low) value of k_ψ after vesicle preincubation at 45°C also proves that NBT does not leak from the vesicle at this temperature. Riboflavin probably penetrates the vesicle at 45°C, because after preincubation with the flavin the values of k_ψ at both 25 and 45°C were higher than those obtained without vesicles. Consequently, MF production after preincubation of NBT-containing vesicles with riboflavin at 45°C may arise because of intravesicular O_2^- production by endovesicular riboflavin.

TABLE I

Effect of temperature on the permeability of DODAC vesicles to superoxide anion produced by riboflavin photolysis

Reaction mixtures contained $1.4 \cdot 10^{-5}$ M riboflavin, 0.002 M methionine, $4 \cdot 10^{-5}$ M NBT in 0.05 M borate buffer (pH 8.2). DODAC concentration (when added) was $1 \cdot 10^{-4}$ M. The first-order constant for monoformazan production from nitroblue tetrazolium reaction with O_2^-, produced by riboflavin photolysis, (k_ψ) was calculated as described in Materials and Methods.

Incubation condition	k_ψ ($s^{-1} \times 10^3$)	
	25°C	45°C
No vesicles	0.75 ± 0.06	1.32 ± 0.2
Vesicles with NBT added externally	0.87 ± 0.06	1.44 ± 0.2
Vesicle-entrapped NBT	0.30 ± 0.02	1.52 ± 0.2
Vesicles containing NBT preincubated 1 h at 45°C	0.01 ± 0.02	–
Vesicles containing NBT preincubated 1 h with riboflavin at 45°C	1.36 ± 0.05	1.94 ± 0.1

TABLE II

Effect of temperature on the permeability of DODAC vesicles to superoxide added as KO_2

Reaction mixtures contained $4 \cdot 10^{-5}$ M NBT in 0.01 M borate buffer pH 9.5, final concentration of DODAC (when present) was $1 \cdot 10^{-4}$ M. Estimated initial superoxide concentration was $2 \cdot 10^{-5}$ M.

Incubation condition	A_{530} of the reaction mixture after addition of KO_2	
	25°C	45°C
No vesicles	0.29 ± 0.04	0.33 ± 0.04
Vesicles with NBT added externally	0.55 ± 0.06	0.51 ± 0.06
Vesicle-entrapped NBT	0.04 ± 0.04	0.37 ± 0.04

Conclusive data showing a striking difference of permeation rates above and below the T_c were obtained by addition of KO_2. Addition of KO_2 to NBT containing vesicles at 25°C did not produce MF, confirming the low O_2^- permeability of DODAC bilayers below the T_c (Table II). Addition of KO_2 to NBT-containing vesicles at 45°C produces MF in yields comparable to those obtained when equivalent concentrations of NBT and KO_2 are incubated in free solution (Table II).

In our system the formation of MF was essentially quantitative after addition of KO_2. Any rate constant higher than 1 s^{-1} would fit this observation and taking this number as a limit we can calculate a lower limit for the permeability coefficient (P) of superoxide anion in DODAC vesicles. The mean diameter of large unilamellar DODAC vesicles is 250 nm and the internal volume is 10 l/mol [15,29]. Hence, the lower limit for the permeability coefficient of superoxide anion through bilayers of DODAC vesicles above the T_c is approx. 10^{-6} cm/s. This estimate is consistent with an estimated value for the O_2^--permeability coefficient of for the phospholipid bilayer in a liquid crystalline phase, e.g., $2.1 \cdot 10^{-6}$ cm/s [9].

These results demonstrate unequivocally that superoxide anion rapidly permeates the bilayer of DODAC

vesicles above the T_c and suggest that DODAC bilayers are little (if at all) permeable to O_2^- below the T_c. The biological implications of this finding are under investigation.

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