Oxygen Diffusion-Concentration in Erythrocyte Plasma Membranes Studied by the Fluorescence Quenching of Anionic and Cationic Pyrene Derivatives[1]

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Abstract

Fluorescence quenching by oxygen of cationic [pyrene-$(CH_2)_n N(CH_3)_3^+$; $n = 1$, 4, and 11] and anionic [pyrene-$(CH_2)_n CO_2^-$, $n = 3$, 8, 11, and 15] probes was investigated in erythrocyte plasma membranes (leaky) in order to assess the ability of oxygen molecules to interact with solutes located at different positions in the membrane. The pseudounimolecular quenching rate constants measured increase, both for cationic and anionic probes, when n increases. These results are interpreted in terms of an increased oxygen solubility toward the center of the membrane interior, and imply that lateral diffusion contributes more than transverse diffusion to total oxygen mobility. For all of the probes considered, quenching rates increase when n-alkanols are added. The effect observed increases when n decreases and when the size of the n-alkanol alkyl chain increases. Arrhenius-type plots for the quenching rate constants show noticeable downward curvatures. Average (0–40°C) activation energies are $\sim 6\,kcal/mol$.

Key Words: Erythrocyte membrane; oxygen penetration; pyrene derivatives; fluorescence quenching in membranes; fluorescence quenching; by oxygen in membranes.

Introduction

Oxygen quenching of fluorescence has been established as a method to determine the oxygen penetration of biological systems (Vaughan and

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Weber, 1970; Lakowicz and Weber, 1973), and it has been lately employed to determine the ability of oxygen to interact with compounds bound to micelles (Turro *et al.*, 1979; Rubio *et al.*, 1985), vesicles and liposomes (Geiger and Turro, 1975, 1977; Abuin *et al.*, 1988; Lakowicz, 1980), and biological membranes (Fischkoff and Vanderkooi, 1975). In particular, Fischkoff and Vanderkooi (1975) employed the quenching of pyrene fluorescence by oxygen to evaluate oxygen diffusion coefficients in erythrocyte plasma membranes (EPM), and reported a value of $7.27 \times 10^{-6} \, cm^2/s$, with a very low (1.1 kcal/mol) activation energy. A relevant factor not addressed in this work is how the oxygen ability to reach a probe is related to its location. In the present work, we report the results obtained in a study of the deactivation of several pyrene derivatives incorporated into EPM by oxygen and discuss the effect that the probe location and *n*-alkanol incorporation have upon the pseudounimolecular interaction rate constant.

Experimental Methods

Excited probe lifetimes were measured by following the fluorescence decay after excitation with a Nitronite nitrogen laser. Fluorescence decays were recorded in a Tektronik 7633 oscilloscope. Fluorescence intensities I were measured from 20 nsec after excitation (I_{20}) to nearly $0.15 \times I_{20}$. The decays were fitted to an equation such as

$$\ln I_{20}/I_t = (\tau^{-1})t$$

and were, inside the precision of the method employed, monoexponential (linear regression coefficient greater than 0.999) both in absence (nitrogen purged) and presence of oxygen. Oxygen saturation or oxygen removal was achieved by gently bubbling oxygen or nitrogen, respectively. Reequilibration with air produced results similar to those obtained under air prior to any bubbling, showing that this treatment does not significantly modify the EPM.

Quenching experiments employing iodide and tryptophan as quenchers were performed by measuring probe lifetimes as a function of the quencher concentration.

Wistar rats were maintained on a standard pellet diet *ad libitum* (Alimentos Balanceados S.A., Santiago). Blood samples were obtained by cardiac puncture using heparinized syringes on rats anesthetized with nembutal (50 mg/kg, i.p.) and were centrifuged at 2300 g for 10 min and 4°C to remove plasma and buffy coats. The cells were washed three times with cold phosphate-buffered saline (PBS) containing 150 mM NaCl and 5 mM sodium phosphate, pH 8.0. Erythrocyte ghosts were prepared by the method

described by Hanahan and Ekholm (1974) and were devoid of hemoglobin. They were diluted with a 5 mM sodium phosphate (pH 8) solution until the working concentration (~ 3 mg protein/ml) was reached. Protein concentrations were determined by the method described by Lowry et al. (1951).

Fluorescent probes (1-pyrenyl)methyltrimethylammonium (PMTMA), 4-(1-pyrenyl)butyltrimethylammonium (PBTMA), 11-(1-pyrenyl)trimethylammonium (PUTMA), 16-(1-pyrenyl)hexadecanoate (PHD), 9-(1-pyrenyl)nonanoate (PN), and 12-(1-pyrenyl)dodecanoate (PD) were from Molecular Probes and were employed as received. Pyrene and 4-(1-pyrenyl)butanoate (PB) were Eastman products and were purified by recrystallization from ethanol. Aliquots of a concentrated methanolic solution were added to the EPM solution in such a way that methanol was $< 0.1\%$ of the total volume and the probe concentration (10^{-6} M) was low enough to make excimer formation negligible. The membranes were incubated for 1 h to assure total probe incorporation. The results obtained were independent of the incubation times for $t \geqslant 40$ min. For the more water-soluble probe employed (PMTMA), its quantitative incorporation to the membrane was assessed by measuring the fluorescence intensity remaining in the ultrafiltrate after ultrafiltration of the EPM solution with the probe through a Millipore membrane.

Results and Discussion

The degree of penetration of the probe into the membrane bilayer can be assessed from quenching experiment employing highly hydrophilic quenchers (Thulborn and Sawyer, 1978). Deactivations by iodide and tryptophan were performed in buffer and in EPM suspensions. Plots of τ^0/τ vs quencher concentration, for most compounds, were linear, and the k_Q values obtained are presented in Table I.

The data in Table I show that all of the probes are protected from deactivation by water-soluble quenchers, and that this protection is less for PMTMA than for the other probes. This result indicates a deeper penetration of these probes toward the membrane interior. The effect of iodide addition upon PB is different than that observed for the other probes, since fluorescence decays become multiexponential. Furthermore, and although in buffer solution K_Q for this compound is almost eight times less than for PBTMA (a result that can be explained in terms of electrostatic repulsion), the effect in the presence of EPM is greater for PB than for PBTMA. EPM is highly asymmetric. In particular, it is relevant to the interpretation of the present results that most of the negatively charged phospholipids are located at the inner half of the lipid bilayer. We can expect then that positively

Table I. Deactivation by Iodide and Tryptophan

| Probe | Quencher | k_Q (10^8 M^{-1} sec^{-1}) | |
		Buffer[a]	EPM suspension[b]
PMTMA	Iodide	35	2.1
	Tryptophon	31	2.8
PBTMA	Iodide	36	$\leqslant 0.08$
	Tryptophon	37	< 0.2
PB	Iodide	5	$\approx 1^c$
PUTMA	Iodide	–	$\leqslant 0.08$
	Tryptophon	–	< 0.2
PN	Iodide	–	< 0.1
PD	Iodide	–	< 0.1

[a] 5 mM sodium phosphate (pH = 8).
[b] In 5 mM sodium phosphate (pH = 8) solution.
[c] Nonlinear decays were obtained in the presence of added iodide.

Table II. Lifetimes Obtained at 37°C

Probe	n	Medium	$\tau_N{}^a$ (nsec)	$\tau_{ox}{}^b$ (nsec)	k_{ox} (10^6 sec^{-1})
PMTMA	1	Buffer	50 ± 1	26 ± 1	18
		EPM	76 ± 1	45 ± 1	9.0
PB	3	Buffer	125 ± 1	42 ± 1	16
		EPM	139 ± 1	46 ± 1	14.5
PBTMA	4	Buffer	97 ± 1	39 ± 1	16
		EPM	205 ± 2	53 ± 1	14
PN	8	EPM	192 ± 2	49 ± 1	15
PUTMA	11	EPM	206 ± 2	39 ± 1	20.5
PD	11	EPM	201 ± 2	48 ± 1	16
PHD	15	EPM	215 ± 3	41 ± 2	20
Py	–	EPM	298 ± 4	62 ± 2	13

[a] Lifetimes measured in nitrogen-purged solutions.
[b] Lifetimes measured in solutions under oxygen at a total pressure of 1 atm.

charged probes are going to be preferentially absorbed at the inner interface, while negatively charged probes will tend to be placed at the outer half of the lipid bilayer. This different distribution could explain the differences observed for the probe–iodide interaction rate when probes bearing different charges are employed. The nonlinear decays observed for PB in the presence of added iodide could be indicative of several probe locations of widely different accessibility to the iodide ions.

Probe lifetimes were dependent upon the oxygen concentration. Typical results obtained at 37°C are presented in Table II, which also includes values of k_{ox}, defined by

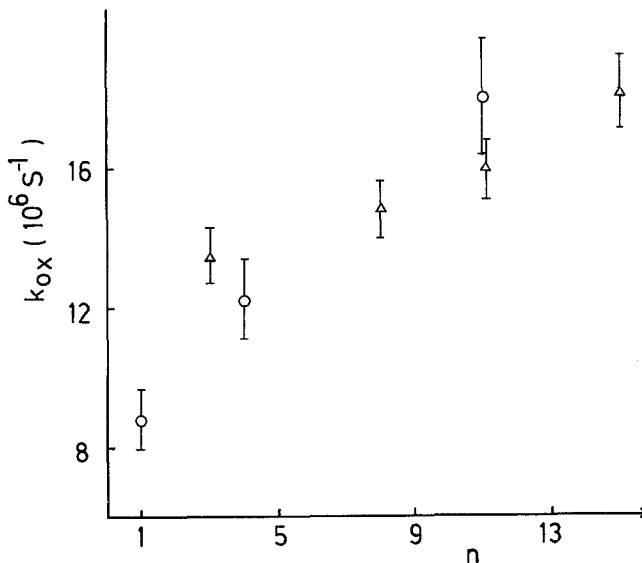$$k_{ox} = \tau_{ox}^{-1} - \tau_N^{-1}$$

Fig. 1. Values of k_{ox} at 37°C plotted as a function of the number of carbon atoms of the alkyl chain: (O) cationic probes and (△) anionic probes.

and that correspond to the pseudounimolecular quenching constnt by oxygen in a solution equilibrated with oxygen at 1 atm total pressure. Since the deactivation process is diffusionally controlled, k_{ox} measures the average time required for an excited probe–oxygen encounter. These values varied appreciably with the membrane preparation and its previous treatment (see the following), but always with a tendency to larger k_{ox} values when n increases were observed (see Fig. 1). A closely similar trend (see Fig. 2) was observed for k_{air} values, defined by

$$k_{air} = (\tau_{air})^{-1} - (\tau_N)^{-1}$$

where τ_{air} is the lifetime measured in air-equilibrated solutions.

The result in Table II regarding the quenching of pyrene by oxygen [and the value of $k_{ox} = 14.4 \times 10^6 \, sec^{-1}$ reported by Fischkoff and Vanderkooi (1975)], would imply that pyrene is deeply incorporated into the membrane. This is in agreement with the rather low value (1.0) measured for the I_I/I_{III} bands of pyrene, that is, indicative of a rather unpolar average environment sensed by the excited pyrene (Dong and Winnik, 1982).

Two remarkable features of the k_{ox} values given in Table II and Fig. 1 are their dependence on the size of the probe alkyl chain, and the fact that k_{ox} values for probes that deeply penetrate the bilayer are very close to those measured in bulk buffer solution. The similarity between k_{ox} values measured
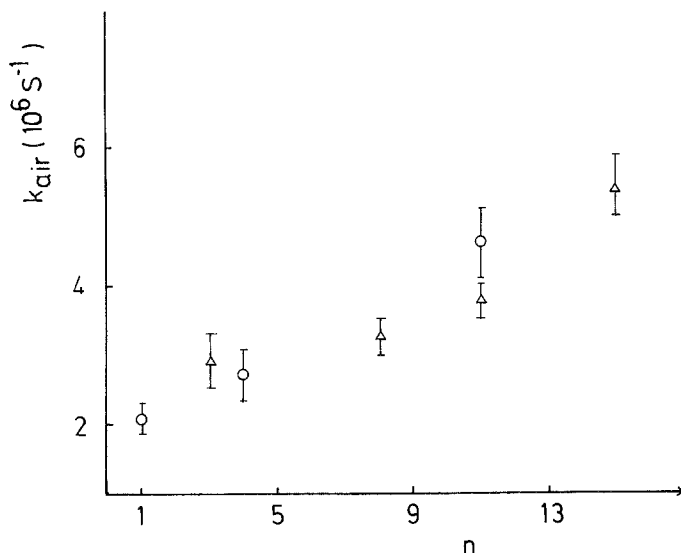
Fig. 2. Values of k_{air} at 37°C: (○) cationic probes and (△) anionic probes.

in buffer and those obtained for probes that can be considered to lie near the center of the bilayer most probably results from a compensation between a higher solubility and a smaller mobility in the EPM (Lakowicz, 1982).

The differences observed between k_{ox} (and k_{air}) values of the different probes can be interpreted in terms of a reduced microviscosity and/or increased oxygen solubility toward the center of the membrane bilayer. We consider that concentration gradients offer a more reasonable explanation than changes in local microviscosity due to the fact that, in the time required for an oxygen probe encounter (50 nsec under oxygen and 200 nsec under air), the oxygen molecules are able to travel over relatively long distances (≈ 70 and 140 Å under oxygen and under air, respectively) and hence the local microviscosity is not a relevant factor. On the other hand, in its random walk through the membrane, the oxygen molecule must travel preferentially through these zones where its average concentration is higher, thus hitting more frequently the probes located there. Our data indicate therefore that the average oxygen "concentration" in the EPM increases toward the membrane center.

The results obtained in the present work emphasize the relevance of transverse oxygen motion in the central regions of the lipidic bilayer, since this type of movement is the only one that can explain the observed dependence of k_{ox} on the probe location. The fluidity of the membrane (in

particular for transverse motion) increases toward the center of the bilayer (Blatt and Sawyer, 1985; Vanderkooi and McLaughlin, 1976). It can be expected then that the solubility of the oxygen must also increase in the same direction. The interplay of both factors could explain the trend observed in the present work. It is also interesting to note that, due to the fact that the oxygen molecule interacting with the excited pyrene has traveled during the excited probe lifetime over relatively long distances, the results obtained in the present work are much less sensitive to local perturbations arising from the probe incorporation into the membranes than those obtained by other methods (i.e., fluorescence depolarization) that only sense the probe microenvironment.

The oxygen diffusion concentration gradient in dipalmitoyl phosphatidylcholine model membranes has been studied by Vachon *et al.* (1987) by an e.s.r.-saturation technique employing spin-labeled stearic acids as probes (with the probe located at positions 5, 7, 10, 12, and 16). At temperatures above the phase transition (the condition tht can be assumed for the EPM at 37°C), the results obtained do not show a clear trend with the spin label position, but the smaller oxygen effect is sensed when the probe is located at the 16 position, a result that contrast with those found in the present work. Also, Tsuchida *et al.* (1984) have reported tht oxygen binding to heme groups in homogeneous solvents occurs nearly 10^3 times faster than with liposome-included hemes presumably located at the center of the lipid bilayer. These results were interpreted in terms of a very reduced rate of oxygen diffusion toward the center of phospholipid membranes. Afzal *et al.* (1986), by measuring proton-spin lattice relaxation rates in egg yolk phosphatidylcholine vesicles, concluded that the terminal methyl group is almost equally exposed to oxygen as the methylene groups. The differences between these results and those obtained in the present work would indicate that lateral diffusion is much more relevant in the EPM than in model phospholipid membranes, even when these systems are above their phase transition temperature.

The membrane characteristics can be modified by incorporation of solutes (Houslay and Stanley, 1982), in particular *n*-alkanols (Rowe *et al.*, 1987; Tristman and Moynihan, 1981). *n*-Alkanol addition does not modify the lifetimes under nitrogen, but considerably decreases the lifetimes measured in the presence of oxygen. This implies that the only effect of the *n*-alkanols is to increase the rate of oxygen quenching (Abuin *et al.*, 1988). Values of k_{ox} measured for the different probes (at 25°C) as a function of *n*-octanol addition are presented in Fig. 3. The data show that k_{ox} increases for all the probes when the *n*-octanol concentration increases. Nevertheless, the relative increase in k_{ox} tends to decrease when the size of the alkyl chain of the probe increases. This is emphasized by the data presented in Table III. This table
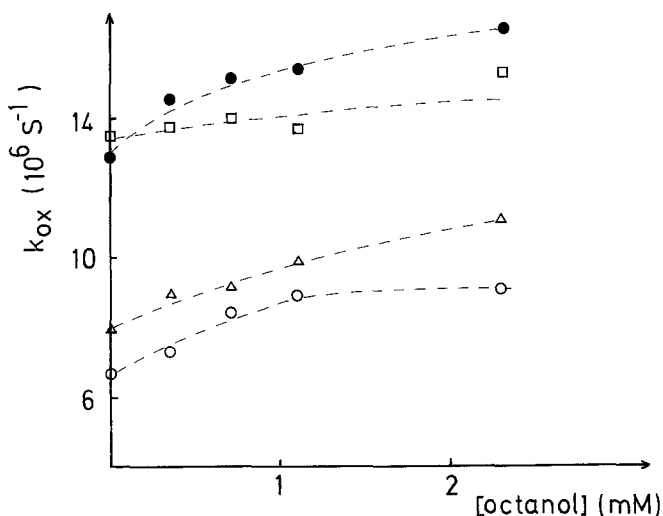
Fig. 3. Values of k_{ox} at 25°C as a function of the n-octanol concentration (protein, 4.4 mg/ml): (O) PMTMA, (△) PBTNA, (⊙) PUTMA, and (□) PHD.

shows that the additive concentration needed to produce significant increases in k_{ox} decreases notably when the size of the alkyl chain of the alkanol increases. Furthermore, the data in Table III also show that the greatest effect of the alkanols is on the probe (PMTMA) located at the interface, and that this selectivity seems to increase when the size of the n-alkanol decreases. The first effect (greatest effect of the higher alkanols) may be partly related to different degrees of incorporation (Rowe et al., 1987). The greatest effect of PMTMA may be related to the fact that this probe is located in the more

Table III. Effects of Alkanols at 25°C

Additive	Probe (n)	k_{ox}/k_{ox}^0 [a]
n-Butanol (100 mM)	PMTMA (1)	1.16
	PBTMA (4)	1.08
	PUTMA (11)	1.0
n-Hexanol (15.6 mM)	PMTMA (1)	1.40
	PBTMA (4)	1.26
	PUTMA (11)	1.18
	PHD (15)	1.05
n-Octanol (2.3 mM)	PMTHA (1)	1.36
	PBTMA (4)	1.39
	PUTMA (11)	1.29
	PHD (15)	1.14

[a]Ratio between the pseudounimolecular quenching rate constant by oxygen (1 atm total pressure) in the presence (k_{ox}) and in absence (k_{ox}^0) of added n-alkanol.
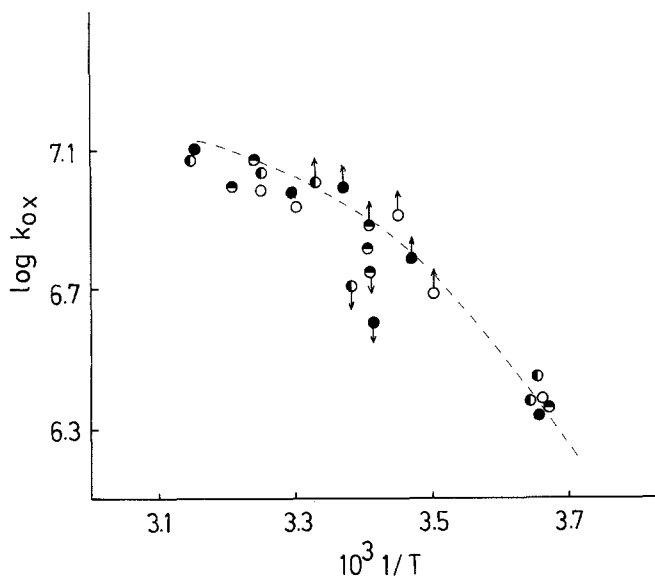
Fig. 4. Values of $\log k_{ox}$ plotted as a function of the inverse temperature (PMTMA as probe). Arrows indicate whether the sample was being heated ($\uparrow$) or cooled ($\downarrow$). Different symbols correspond to different samples.

rigid part of the membrane (and thus that most sensitive to additives) and that the alkanols will be preferentially solubilized in this region due to the polarity of the hydroxyl group that will tend to remain at the membrane interface.

The change in k_{ox} with temperature in the 0–40°C range is shown in Figs. 4 and 5 for PMTMA and PUTMA, respectively. The data show, particularly for PMTMA, a noticeable downward curvature and hysteresis (i.e., values obtained when the sample is being heated are higher than when the measurements are carried out in cooling experiments) although the samples were incubated by nearly 15 min at each temperature prior to the measurements. Similar results were obtained for the other pyrene derivatives (data now shown). This makes it difficult to interpret the temperature effect upon the interaction rate. Nevertheless, the lack of a transition temperature as that observed in synthetic vesicles and liposomes (Fischkoff and Vanderkooi, 1975; Abuin *et al.*, 1988), and the great temperature dependence of the quenching rate, are remarkable. The lack of a transition temperature can be explained in terms of the membrane heterogeneity. The dispersion of the data and the observed hysteresis make it difficult to obtain an "activation energy" for the process. Nevertheless, the "average" activation energies between the extreme temperatures considered are nearly 6.5 kcal for PMTMA and 5 kcal
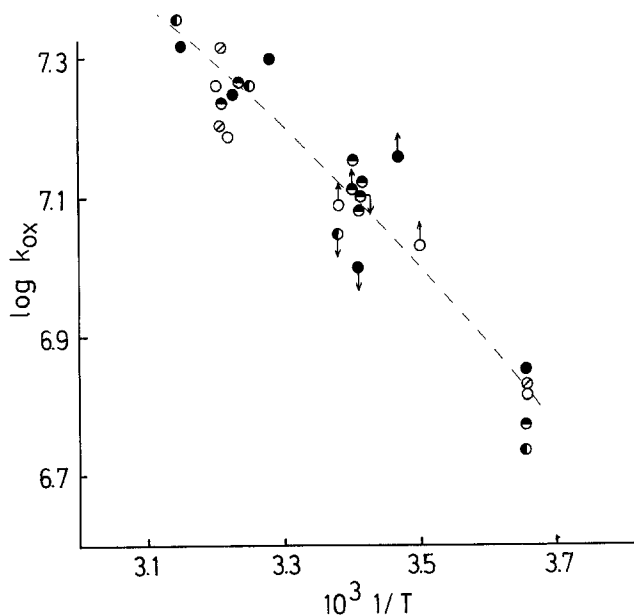
Fig. 5. Values of $\log k_{ox}$ as a function of the inverse temperature (PUTMA as probe).

for PUTMA. These values are considerably higher than that measured for PMTMA in water (2 kcal/mol) and stress the fact that oxygen diffusion in the aqueous phase is not the rate-limiting step. Fischkoff and Vanderkooi (1975) reported a considerably smaller value for the activation energy for pyrene quenching by oxygen in EPM (1.1 kcal/mol). We consider that the difference arises from a different treatment of the experimental data. If values of k_{ox} are calculated and plotted as in Figs. 4 and 5, the data previously reported render an activation energy of 5.7 kcal, a value in the same range as those obtained in the present work. This activation energy is of the order expected for a diffusion-controlled process in a viscous hydrocarbon media. Nevertheless, in the present system, it must reflect both the changes in oxygen mobility and solubility within the bilayer.

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