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Oxygen Gradients in Mitochondria Examined with Delayed Luminescence from Excited-State Triplet Probes†

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Received November 21, 1989; Revised Manuscript Received March 15, 1990

ABSTRACT: Phosphorescent probes are described that are quenchable by dioxygen and that partition into membranes. These probes are derivatives of porphyrin, in which the central metal, either zinc or palladium, induces intersystem crossing to enhance the triplet yield. The location of the probe in a suspension of membranes depends upon the charge distribution of side groups on the porphyrins. Probes that partition into the membrane are sensitive to phase transitions in lecithin artificial membranes. In the mitochondria these membrane probes are within transfer distance from tryptophans in membrane proteins. Although absolute concentrations of oxygen in membranes cannot be determined by this method, by comparing the oxygen dependence of a probe in the aqueous phase with that in the membrane phase under actively respiring and inhibited conditions, it is possible to examine the extent of O_2 depletion at the mitochondrial surface. We show that at oxygen tensions of 0.2 μM and higher these gradients are insignificant at usual oxygen consumption rates of mitochondria.

Oxygen-consuming reactions in cells are localized in mitochondria and microsomes. It follows that there should be a drop in O_2 tension at the membrane surface of these organelles, the size of which will be determined by the rate of oxygen utilization by the relevant reactions and the rate that it is replenished via diffusion. Opinions on the magnitudes of such gradients differ sharply. Experiments from some laboratories [Gayeski & Honig, 1986; Wittenberg & Wittenberg, 1985; Wilson et al., 1988; for a review see Wittenberg and Wittenberg (1989)] indicate that the pressure drop surrounding isolated mitochondria is very small, less than 0.2 Torr (0.32 μM), whereas other studies (Jones, 1986; Tamura et al., 1978; Williamson & Rich, 1983) suggest very steep gradients, as high as several Torr, in cardiac myocytes. This apparent discrepancy could be resolved by direct measurement of $[O_2]$ in the medium surrounding the mitochondrion and in the interior of the mitochondrial membrane. In this work we have used hydrophobic molecules with long-lived delayed luminescence (delayed fluorescence and phosphorescence) to monitor $[O_2]$ in the lipid bilayer whereas water-soluble probes were employed to determine O_2 tension in the bulk phase of the medium.

Because delayed luminescence is quenched only by those O_2 molecules that collide with the sensor during its excited-state lifetime, i.e., those in the immediate vicinity of the probe, local oxygen levels can be sensitively reported. By using two probes in different locations with distinguishable emission properties, differences in oxygen concentration in the two respective sites can be monitored. The purpose of this study was to design the appropriate probes, to examine their O_2-sensing properties, and to use them to measure the O_2 drop across the mitochondrial membrane. By comparing the O_2 dependence of the two sensors under actively respiring and inhibited conditions, it was possible to examine the significance of an O_2 depletion at the mitochondrial surface at tensions of 0.2 μM and higher.

Experimental Procedures

Materials. Chemicals were obtained as follows: coproporphyrin and mesoporphyrin derivatives from Porphyrin

†This work was supported by NIH Grant GM 36392.
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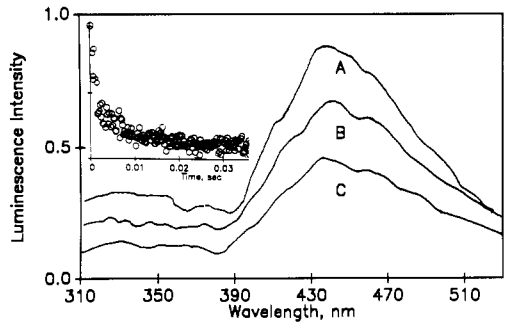

FIGURE 1: Spectra of tryptophan phosphoresence of mitochondria. Mitochondria (0.25 mg of protein/mL) were suspended in 225 mM mannitol, 75 mM sucrose, 0.5 mM EDTA, and 5 mM MOPS, pH 7.4. Excitation, 283 nm; delay times, 0.5 ms (A), 2.0 ms (B), and 7.0 ms (C); gate time, 2.0 ms. Inset: Decay of luminescence after lamp flash. Excitation, 283 nm; emission, 440 nm.

Products, Inc., Logan, UT; glucose oxidase (type II, β-D-(+)glucose:O_2 oxidoreductase, E.C. 1.1.3.4), catalase (bovine liver hydrogen-peroxide:hydrogen-peroxide oxidoreductase, E.C. 1.11.1.6), horse myoglobin and dimyristoylphosphatidylcholine from Sigma Chemical Co. (St. Louis, MO); and sodium amytal (amobarbital) from Eli Lilly Inc. (Indianapolis, IN). Carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP)[1] was a gift from Dr. Peter G. Heytler (Du Pont, Wilmington, DE).

Preparation of Methyl-Substituted Myoglobins. The butanol extraction method of Teale (1959) was used to remove the heme from myoglobin. The desired metal porphyrin, dissolved in dimethylformamide, was added dropwise to the solution of apomyoglobin that was adjusted to pH 8.6 with ammonium hydroxide. At least 30 s was allowed between addition of the drops. The sample was kept on ice for 45 min with stirring, followed by dialysis and Sephadex G-25 chromatography to remove excess metal porphyrin (Vanderkooi et al., 1985).

Instrumentation and Analysis. Absorption spectra were measured on a Perkin-Elmer 200 spectrophotometer. Prompt fluorescence spectra and delayed luminescence spectra were obtained on a Perkin-Elmer LS-5 spectrofluorometer. Phosphorescence lifetimes were determined on a lifetime instrument described previously (Green et al., 1987). All instruments were equipped with red-sensitive Hammamatsu 928 photomultiplier tubes. Phosphorescence decay data are fit by nonlinear analysis to a single- or double-exponential function, with the goodness of fit being expressed in terms of a correlation coefficient.

Membrane Preparation. Phospholipid vesicles were prepared by sonication of the lipids with a Heat Systems-Ultrasonics Inc. sonifier for 30 s at high power. Mitochondria were isolated from the livers of male Sprague-Dawley rats, 200–250 g, by the procedure of Schneider (1948). The isolation medium consisted of 0.225 M mannitol, 0.075 M sucrose, 0.0005 M ethylenediaminetetraacetic acid (EDTA), and 0.005 M 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.4. All experiments were performed in the same medium. The oxygen consumption rate of the mitochondria was measured by using a Clark electrode. Mitochondrial protein was determined by the biuret method.

Results

Intrinsic Phosphorescence of Mitochondria. The possibility of using phosphorescence of mitochondrial tryptophan(s) as an intrinsic oxygen sensor was first explored (Figure 1). The emission maximum for fluorescence of the mitochondrial suspension was at 347 nm under anaerobic conditions. No phosphorescence could be detected from conventional steady-state emission spectra of mitochondria at room temperature. However, when a delay was introduced to eliminate the contribution of prompt fluorescence, delayed emission at 445 nm, typical of tryptophan phosphorescence, was observed. The phosphorescence intensity decreased when air was introduced to the sample and no signal was detected in air-saturated buffer. The decay of phosphorescence was non single exponential. A decay component with a lifetime of about 7 ms is shown on the inset of the figure. A shorter component with a lifetime of ~0.5 ms and a longer component of about 30 ms were also identified (not shown). The complex decay behavior of tryptophan, arising from the many proteins in mitochondria, made unique analysis of the decay curve impossible and rendered it impractical to use the intrinsic phosphorescence of this amino acid as an oxygen sensor. However, the observation that the phosphorescence was quenched at ambient oxygen levels indicates that these protein tryptophans are accessible to oxygen.

Characteristics of Hydrophilic and Lipophilic Phosphorescent Derivatives of Porphyrin. In order to be able to measure oxygen independently in the aqueous phase and in the membrane, the probes must be located in different positions and their emission spectra must be well separated so that a signal from each can be uniquely measured. Using the same parent chromophore, porphyrin, these two requirements can be met. Distinct emission spectra can be obtained by altering the central metal while the probe hydrophobicity and charge, and therefore its partition behavior, can be varied by changing the side groups on the ring. Coproporphyrin with four negative charges on opposite sides of the aromatic ring is water soluble; mesoporphyrin with two negative charges on the same side of the ring is expected to be amphoteric and, therefore, would partition into the lipid/aqueous interface of membranes. In addition, metal derivatives of this compound resemble the native heme, and it is known that they can be incorporated into the heme pocket of apomyoglobin (Vanderkooi et al., 1985).

The spectra of Zn coproporphyrin and Pd coproporphyrin in buffer and of Pd mesoporphyrin and Zn mesoporphyrin in suspensions of dimyristoylphosphatidylcholine are shown in Figure 2A,B. For comparison the Zn and Pd mesoporphyrin derivatives of myoglobin (Zn MP myoglobin and Pd MP myoglobin, respectively) are shown in Figure 2C. The emission maximum for the Pd porphyrins was at 666 nm. The emission peaks of the Zn porphyrins were at 582 and 635 nm; these peaks represent delayed fluorescence that arises from the repopulation of the singlet excited state from the triplet state by thermal activation, while the third peak at 708 nm is attributed to phosphorescence (Feitelson & Mauzerall, 1982; Dixit et al., 1984). The spectra were remarkably the same for the particular metal derivative, irrespective of the environment. However, the emission bandwidths were somewhat smaller in the case of the Pd derivatives bound to dimyristoylphosphatidylcholine (Figure 2B) or myoglobin (Figure 2C) as compared with coproporphyrin in aqueous solution (Figure 2A). It can be seen that using 580–582 nm

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MP, mesoporphyrin; MP myoglobin, myoglobin in which the heme was replaced with mesoporphyrin; Pd MP myoglobin, myoglobin in which the heme was replaced with palladium mesoporphyrin.

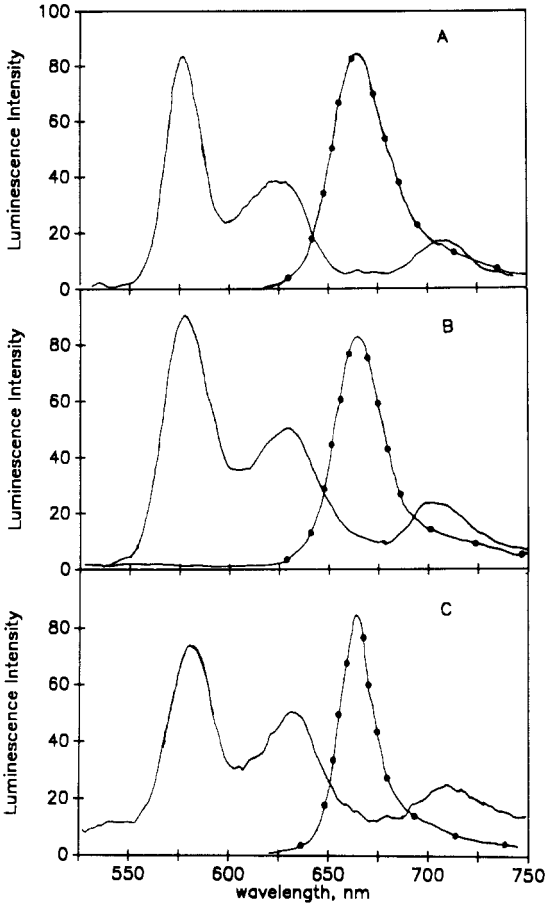

FIGURE 2: (A) Delayed luminescence emission spectra of Zn coproporphyrin (—) and Pd coproporphyrin (●) at 22 °C, (B) Zn mesoporphyrin (—) or Pd mesoporphyrin (●) in 1 mg of dimyristoylphosphatidylcholine/mL at 15 °C, and (C) Zn myoglobin (—) or Pd myoglobin (●) at 20 °C. The medium for all samples was 0.1 M NaCl and 0.02 M Na phosphate, pH 7.0.

Table I: Phosphorescence Lifetimes in the Absence of Oxygen

substance	lifetime (ms)	temp (°C)	k_q (M^{-1} s^{-1})
Pd coproporphyrin[a]	0.66	20	3.8×10^9
Zn coproporphyrin[a]	0.9	20	2.8×10^9
Pd mesoporphyrin in lecithin[b]	1.3	15	
	1.0	20	
	0.4	30	
Zn mesoporphyrin in lecithin[b]	10	15	
	6.5	20	
	0.5	30	
Pd MP myoglobin[b]	1.2	20	9.7×10^7
Zn MP myoglobin[b]	12	20	1×10^8
Pd mesoporphyrin (mitochondria)[c]	0.9	25	
Zn mesoporphyrin (mitochondria)[c]	5.1	25	$(2.3 \pm 1) \times 10^8$

[a] Lifetime was measured at a dye concentration 10 nM. The value of k_q for Pd coproporphyrin was determined by Vanderkooi et al. (1987) and for Zn coproporphyrin carried out as described in Figure 1. [b] Conditions given in the caption of Figure 2. [c] Conditions given in the caption of Figure 6.

as detection wavelengths the emission of the Zn porphyrin triplet state can be measured independently of the Pd porphyrin emission. There is a small contribution of the Zn emission to the Pd emission with 666 nm as the emission maximum, but by adjusting the excitation wavelength and the concentration, it can be made to be less than a few percent.

The delayed luminescence lifetimes of the probes in the absence of oxygen are given in Table I. The lifetimes were

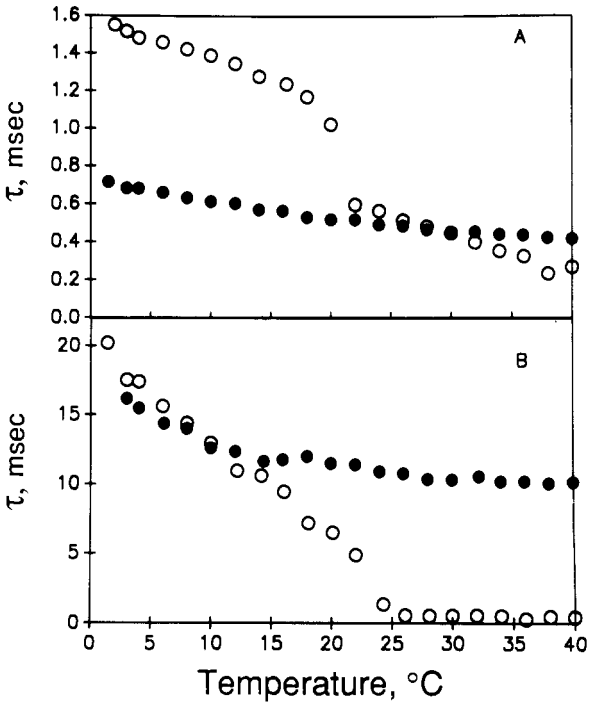

FIGURE 3: Temperature dependence of phosphorescent probes in the presence of 1 mM dimyristoylphosphatidylcholine in 0.1 M NaCl and 20 mM phosphate buffer, pH 7: (A) Pd coproporphyrin (●) and Pd mesoporphyrin (○); (B) Zn Mp myoglobin (●) and Zn mesoporphyrin (○).

independent of wavelength chosen for emission, irrespective of whether delayed fluorescence or phosphorescence was being measured. This is a characteristic of thermally activated delayed fluorescence in which equilibrium occurs in the excited state (Parker, 1967). The triplet-state lifetimes of the Pd porphyrins are relatively insensitive to environment; for the coproporphyrin in aqueous solution the lifetime was 0.66 ms, whereas for Pd mesoporphyrin in lecithin or for Pd mesoporphyrin in myoglobin the lifetime was 1.0 and 1.2 ms, respectively, at 20 °C. A greater sensitivity to environment was seen for the Zn derivatives. In the absence of oxygen, the lifetime of Zn coproporphyrin in aqueous medium was 0.9 ms, which is considerably less than the lifetime of Zn mesoporphyrin in lecithin (6.5 ms) or in the protein (12 ms).

Effect of Membrane Phase Transition on Membrane Probes. Although Pd and Zn mesoporphyrin luminescence yields and lifetimes are sensitive to O_2 in membranes, these parameters may also respond to other factors of the membrane environment. As evidence for this, in the absence of O_2 the temperature dependence of the phosphorescence lifetime of Pd mesoporphyrin and Zn mesoporphyrin in dimyristoylphosphatidylcholine showed discontinuity at around 23 °C, the main phase transition temperature of the lipid, and at ~12 °C, the pretransition temperature (Figure 3). The lifetime for Pd mesoporphyrin in the lipid was 1.3 ms at 10 °C, compared with 0.4 ms at 30 °C. The emission of Zn mesoporphyrin in lipid showed more variation than that of Pd mesoporphyrin in intensity in the temperature range of the lipid pretransition (Figure 3B). In contrast to the temperature dependence of these probes in lipids, Pd coproporphyrin in buffer and Zn MP myoglobin showed a continuous decrease in lifetime as a function of temperature, which indicates that the luminescence from the porphyrins themselves does not exhibit temperature discontinuity. Furthermore, their intensities and lifetimes were independent of the presence of lipid. This is consistent with the supposition that neither Pd co-

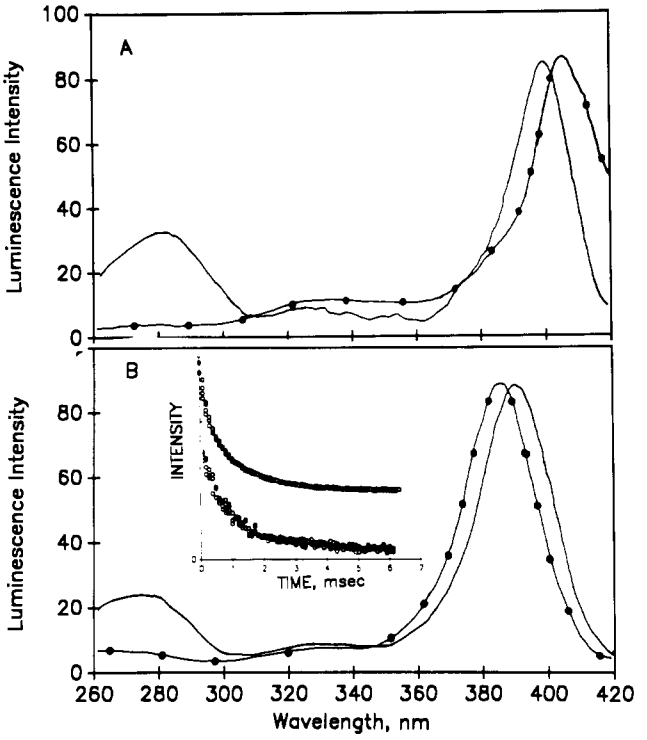

FIGURE 4: (A) Excitation spectra of Zn mesoporphyrin (—) and Zn coproporphyrin (●), both ~50 nM, using 580 nm as the emission wavelength; (B) excitation spectra of ~20 nM Pd mesoporphyrin (—) and Pd coproporphyrin (●) using 666 nm as the emission wavelength. All samples contained 0.5 mg/mL mitochondrial protein in mannitol, sucrose, EDTA, and MOPS, as in Figure 2. Inset: Decay of Pd mesoporphyrin with an excitation wavelength of 280 nm (lower) or 390 nm (upper).

proporphyrin nor Zn MP myoglobin interacts with the lipid under these conditions.

Binding of Probes to Mitochondria. The data presented above suggest that metallo derivatives of coproporphyrin and myoglobin do not bind significantly to phospholipid vesicles, whereas the derivatives of mesoporphyrin do. A similar result would be expected for binding to mitochondria. This was tested by incubating the mitochondria with the respective probe, followed by centrifugation and measurement of free probe level in the supernatant fraction. For Pd coproporphyrin, the binding to mitochondria was insignificant.

Mesoporphyrin derivatives showed binding to mitochondria as indicated by fluorescence being associated with the pellet and not the supernatant of centrifuged suspensions of mitochondria (data not shown). But because the mesoporphyrin in the absence of mitochondria tended to self-aggregate and associate with glass, exact quantitation of the binding was not made by analysis of the supernatant. However, confirmation that bound compound contributes to the phosphorescence signal was obtained from an analysis of the phosphorescence excitation spectra recorded in the presence of mitochondria. The results for Zn coproporphyrin and Zn mesoporphyrin are presented in Figure 4A and those for Pd derivatives are shown in Figure 4B. It can be seen that with mitochondria present there was an increase in excitation for the mesoporphyrin derivatives in the spectral region of 280 nm, where the aromatic amino acids absorb, as compared to coproporphyrins, the probes that do not bind.

The characteristic excitation peak at 280 nm indicates that energy transfer from the tryptophan excited state to the bound mesoporphyrin derivative is occurring. For singlet–singlet energy transfer, the effective distance is 4–5 nm (Forster, 1948), which means that only Pd mesoporphyrin molecules

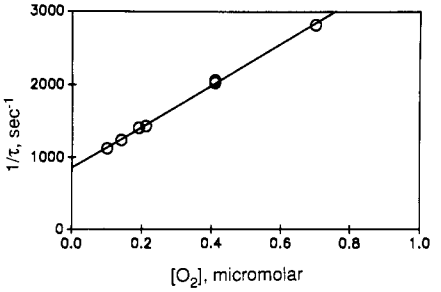

FIGURE 5: Oxygen quenching for Zn coproporphyrin on the basis of Pd coproporphyrin as the oxygen probe. Medium contained 10 nM Pd coproporphyrin and 50 nM Zn coproporphyrin in 100 mM NaCl and 20 mM phosphate buffer, pH 7.0. Temperature: 20 °C. Excitation and emission wavelengths for Zn coproporphyrin were 420 and 580 nm and for Pd coproporphyrin 390 and 667 nm, respectively. Data were fit to a straight line by linear regression.

within this distance, i.e., only those bound to the membrane, would accept energy from tryptophan. If so, the data would indicate that the mesoporphyrin but not the coproporphyrin is bound.

Additional evidence that only bound Pd mesoporphyrin molecules contribute to the phosphorescence comes from the decay profiles. Independent measurement of the phosphorescence lifetime of Zn or Pd mesoporphyrin (at 20 nM) in aqueous buffer gave a lifetime value of <300 μs, i.e., very short. The decay properties of Pd mesoporphyrin phosphorescence were recorded in the presence of mitochondria by using as excitation light at 275 nm (excitation of the tryptophan) or at 395 nm where only the porphyrin is excited (Figure 4B inset). The decay curves and lifetimes were essentially the same. Because the lifetime of tryptophan phosphorescence contains components longer than the Pd mesoporphyrin (see Figure 1), the identical decay behavior at the two excitation wavelengths suggests that energy transfer occurs from the excited singlet state of the mitochondrial tryptophan to the porphyrin. The data on the spectra and decay profiles taken together show that contribution from unbound, if any exists, mesoporphyrin derivative to delayed luminescence is negligible and that it thus serves as a sensitive probe of O_2 in membranes.

Measurements of $[O_2]$ *Using Phosphorescence Probes.* Oxygen concentration can be calculated from delayed luminescence according to the equation of Stern and Volmer (1919), modified for lifetimes, as follows:

$$\tau_0/\tau = 1 + \tau_0 k_q[O_2] \quad (1)$$

where the experimentally determined bimolecular rate constant for quenching is k_q and τ_0 is the lifetime in the absence of quencher.

For Pd coproporphyrin the value of k_q in the aqueous phase was determined by equilibrating the solution with known O_2 gas mixtures (Vanderkooi et al., 1987a). Once this value was known, Pd coproporphyrin was used as a standard to calibrate the response of the other probes. Oxygen was varied by slow removal using glucose as a reductant in the coupled enzyme system containing glucose oxidase and catalase. The phosphorescence lifetime of Pd coproporphyrin was used to determine the oxygen concentration and the k_q of the unknown sample calculated from its lifetime according to eq 1.

An example of the standard quenching plot used for calibration is shown in Figure 5. If the oxygen quenching constant of Pd coproporphyrin is taken as 3.8×10^9 M^{-1} s^{-1} (Vanderkooi et al., 1987a), the quenching constant of Zn coproporphyrin was found to be 2.8×10^9 M^{-1} s^{-1} and for Zn and Pd myoglobin 1×10^8 M^{-1} s^{-1}.

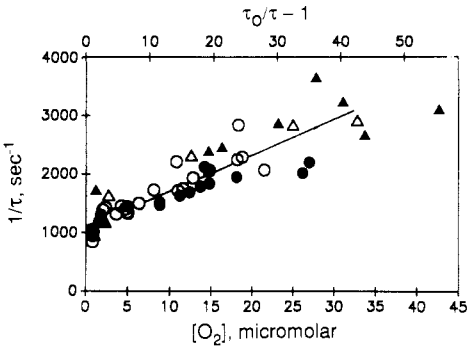

FIGURE 6: Oxygen response of phosphorescent probes, 10 nM Pd mesoporphyrin and 10 nM Zn MP myoglobin, in the presence of mitochondria under respiring (open symbols) and nonrespiring conditions (closed symbols, the sample contained 2 mM amytal): (●, ○) 0.5 mg of mitochondrial protein/mL; (▲, △) 0.06 mg of protein/mL. Buffer was 0.225 M mannitol, 0.075 M sucrose, 0.5 mM EDTA, 2 μM FCCP, and 5 mM MOPS, pH 7.0. Temperature: 25 °C. Excitation and emission wavelengths for Zn mesoporphyrin were 405 and 580 nm and for Pd MP 390 and 666 nm, respectively. The sample was stirred during the measurement.

Phosphorescent Lifetimes in Mitochondria as a Function of Oxygen Consumption. During respiration, there should be a local depletion of oxygen in the mitochondria, and therefore for a given oxygen concentration in the aqueous phase, the lifetime of the phosphorescent probe situated in the membrane should be longer when mitochondria respire actively than in the absence of O_2 consumption.

The phosphorescence lifetimes of Zn MP measured in respiring mitochondria and in the presence of amytal were plotted as a function of O_2 concentration in the medium, as calculated from the quenching of Pd coproporphyrin (Figure 6). The data show that the points obtained from respiring and nonrespiring mitochondria fall on the same straight line. This means that there is no difference in O_2 concentrations between the medium and the membrane down to ≤ 1 μM external O_2. The data were essentially the same for stirred and unstirred samples (not shown). Under anerobic conditions the value of τ_0 for membrane-bound probes was the same for the mitochondria with or without amytal. This shows that amytal itself has no large effect on the membrane that would alter the properties of the dye.

Discussion

The purpose of this study was to characterize phosphorescent molecules that are suitable as probes for O_2 in cellular membranes. Oxygen itself does not have a spectroscopic signal that allows for its direct determination; however, several indirect techniques to measure oxygen distributions in cells and tissues have been devised. For example, intrinsic NADH fluorescence has been used to locate anoxic regions in perfused heart (Steenbergen et al., 1977). This method, however, has been criticized for lack of standardization for quantitative determination of oxygen (Katz et al., 1988). The absorption of intracellular cytochromes can also be used since they become reduced in anoxia (Chance et al., 1973; Tamura et al., 1978), but like NADH fluorescence, the absorption changes cannot be quantitatively related to oxygen pressure in part because they do not depend only on oxygen pressure, but they depend on other variables such as cellular reducing potential and energy level (Wilson et al., 1979). More successful optical methods have been based on measurements of hemoglobin (Kekonen et al., 1987) or myoglobin oxygenation (Fabel & Lübbers, 1965; Tamura et al., 1978). Gayeski and co-workers (Gayeski & Honig, 1986; Gayeski et al., 1987) have measured myoglobin oxygenation by microspectrophotometry in sections of frozen muscle tissue. This has high spatial resolution but is limited to muscle tissue.

Oxygen sensing can be achieved by using extrinsic molecular probes with optical or magnetic properties that are affected by the presence of oxygen. Fluorine-19 has been used to monitor oxygen in biological systems (Joseph et al., 1985; Taylor & Deutsch, 1983). Morse and Schwartz (1985) and Hyde and co-workers (Subczynski & Hyde, 1983, 1984, Subczynski et al. 1989; Yin et al., 1987) utilized the oxygen-dependent line broadening of spin-label probes to monitor oxygen in cells and membranes. Magnetic relaxation techniques have some advantages, especially in turbid samples, but suffer from a limited range of sensitivity, lack of easy imaging capability, and in the case of the spin-label probes, probe chemical instability (Swartz et al., 1986).

Optical probes have an advantage over magnetic probes in terms of sensitivity. Most optical excited states involving π electrons are quenchable by dioxygen, but since sensitivity to oxygen increases with the lifetime of the probe, long-lived probe molecules have to be used at low O_2 concentrations. Pyrene has one of the longest lived excited singlet states of all molecules with a lifetime of ~500 ns and its derivatives have been employed to measure oxygen concentrations in tissues (Knopp & Longmuir, 1972; Opitz & Lübbers, 1984; Benson et al., 1980) and in membranes (Fischkoff & Vanderkooi, 1975). Still greater sensitivity can be achieved by using delayed luminescence (phosphorescence or delayed fluorescence) observed from excited triplet state molecules [for a review, see Vanderkooi and Berger (1989)].

In principle, tryptophan could be employed as an intrinsic probe of oxygen because phosphorescence of this amino acid in proteins is quenched by oxygen (Calhoun et al., 1983). Mitochondria show intrinsic phosphorescence in the absence of oxygen, which is identified by its characteristic spectrum as arising from tryptophan (Figure 1). However, because mitochondria contain so many different proteins and most proteins exhibit phosphorescence (Vanderkooi et al., 1987b), it was not possible to identify the origin of the emitting tryptophan(s). Long phosphorescence lifetime at room temperature is characteristic of buried tryptophans in rigid environments (Strambini & Gonnelli, 1985; Papp & Vanderkooi, 1989), and therefore tryptophan phosphorescence is an indication that some tryptophans in mitochondria are in such an environment.

To measure oxygen at the mitochondrion, extrinsic probes were added to the membrane. Soluble phosphorescent probes for oxygen have been characterized in detail previously (Vanderkooi et al., 1987a), but membrane probes have not. In this work we have developed such sensor molecules and have shown that the phosphorescence yield and lifetime of these probes are sensitive to the phase transition in lipids, changing dramatically at the phase transition temperature (Figure 4). The change in lifetime at the transition temperature could be due in part to increased accessibility of contaminating quenchers in the solution that quench by collisions; the ground-state porphyrins themselves can react with excited states to quench. It is also well-known that porphyrins can form ground-state complexes with a variety of molecules and can self-aggregate (Mauzerall, 1965); such complexes can cause spectral shifts and lifetime changes. An effect of viscosity may also be a direct contributing factor since, in general, out-of-plane distortions cause mixing of the singlet and triplet states and increase the radiationless transition (Lower & El-Sayed, 1966). Whatever the reasons, phosphorescence yields and lifetimes at room temperature are exquisitely sen-

sitive to the rigidity of the environment. It is noteworthy that the Zn MP derivative appears relatively more sensitive than Pd MP to the phase transition of dimyristoylphosphatidylcholine. One reason for this may simply be that the lifetime of the Zn derivative is longer than that of the Pd derivative and the longer the lifetime the larger the chance that the excited state molecule will be influenced by neighboring molecules. However, since the Zn porphyrin derivatives can ligate at the fifth position, perhaps they are more susceptible to out-of-plane distortions. In contrast, Pd has no extra orbitals for ligation, and it is expected that the ring tends to remain in-plane. Because of the sensitivity of the lifetime to the environment, the phosphorescence lifetime in the absence of specific bimolecular quenching processes can be taken as a parameter related to membrane "fluidity". For mitochondria the phosphorescence lifetimes of Pd mesoporphyrin and Zn mesoporphyrin at 25 °C were 0.9 and 5.1 ms, respectively, in oxygen-depleted medium (Table I). These values are intermediate between the values of the lipid below and above the phase transition and probably reflect the intermediate condition of the mitochondrial lipids between the fully fluid and fully crystalline state.

Phosphorescent probes that are both in the aqueous solution and membrane bound are quenched by oxygen. The oxygen sensitivity for the aqueous probes can be calibrated by equilibration with known amounts of oxygen, and therefore their lifetimes can be used to determine concentrations of O_2 in the medium. However, this same method cannot be used to measure the O_2 concentration within the membrane for the following reason. The value of k_q is related to concentration of the quencher and the diffusion coefficients of the probe, D_d, and of the quencher, D_q:

$$k_q = 4\pi N_p(D_d + D_q) \times 10^3 \quad (2)$$

where N is Avogadro's number and p is a factor that relates the probability that each collision causes quenching (Smoluchowski, 1917). In our case, the diffusion of the probe is much slower than that of oxygen. Therefore, k_q will be essentially directly proportional to the diffusion of oxygen only. By insertion of eq 2 into eq 1 it can be seen that the observed decrease in lifetime (i.e., quenching) is a function of both the concentration of the quencher and its diffusion coefficient. Because we have two unknowns, the absolute value of oxygen concentration in the membrane is not determined. The value of k_q of the membrane probe is within a factor of ~ 10 of that for the soluble probes (Table I). On the basis of the solubility of oxygen in hydrophobic compounds, it has been suggested that oxygen is more soluble in membranes than in water (Fischkoff & Vanderkooi, 1974). No rigorous determination of oxygen partition coefficients into mitochondrial membranes has been made, but Subczynski and Hyde (1983) found that the oxygen partition coefficient into phospholipid membranes above the phase transition was greater than 1. While determination of k_q does not allow us to distinguish between the oxygen concentration and diffusion coefficient in the membrane, k_q, being a constant describing a bimolecular reaction of oxygen, does tell us about the reactivity of oxygen in the membrane. This parameter is not greatly reduced by the membrane, and hence we are able to conclude that the mitochondrial membrane does not impose a significant barrier for oxygen diffusion. A similar conclusion for artificial membranes was obtained by Subczynski et al. (1989) using spin-label probes at much higher oxygen concentrations.

Although we did not determine the value of the oxygen concentration in the membrane, we were able to accurately determine changes in the oxygen concentration between the aqueous and membrane phases. Our results provided no evidence of local depletion of O_2 under respiring conditions and estimate that the drop must be smaller than the error of measurement of about 1 μM.

Several workers have discussed in detail the expected oxygen drop at the mitochondrial surface, assuming spherical (Boag, 1969) or ellipsoid (Clark & Clark, 1985) geometry. For a respiratory rate of (10^{-18} mol of O_2/s)/mitochondrion, a diffusion coefficient for oxygen of 10^{-5} cm^2 s^{-1}, and a mitochondrial radius of 1 μm (10^{-6} cm), the difference between the bulk phase and the surface is 0.4 μM (0.25 Torr). Thus our results are consistent with the prediction based upon the diffusion coefficient of oxygen and its rate of consumption in that they show that there is no significant gradient of oxygen concentration under usual measuring conditions of oxygen concentration from ambient pressures down to 1 μM (0.6 Torr). Degn and Wohlrab (1971) and Wilson et al. (1988) measured an apparent $P50$ value for uncoupled mitochondria respiring at a rate of (1.66 nmol/mg of protein)/s to be less than 0.1 μM. At this O_2 concentration essentially every collision between cytochrome oxidase and oxygen will be effective in electron transfer and the oxygen dependence of respiration, i.e., apparent k_m, will depend upon the rate of oxygen consumption.

By using two probes in two locations we are able to resolve oxygen concentrations at different sites in space. A point should be made concerning the spatial resolution of the technique. Resolution is related to the lifetime. For a probe with a lifetime of 1 ms and for a diffusion coefficient, D, of 10^{-5} cm^{-1} s^{-1}, the radius, r, sampled within one lifetime will be $\sim 10^{-4}$ cm [$r = (2D\tau)^{1/2}$]. For the soluble probe this will be the resolution of the technique. If the probe is fixed, as for the membrane-bound probe, then it tests the oxygen concentration only at that fixed point. Since at all times the probe senses oxygen at its immediate location, the membrane-bound probe will reflect oxygen only in the membrane. This argues against the notion that somehow the membrane probe is oriented on the surface such that it senses oxygen only in the aqueous phase. Likewise, if there is depletion of oxygen within the mitochondrion, the membrane-bound probe will sense this only if the oxygen concentration at the membrane is decreased.

In summary, we have described phosphorescent probes that can be used as membrane sensors for oxygen at low concentrations. By using molecules with different spectra, spatial distributions of oxygen can be obtained on the subcellular level and it can be shown that the gradient around respiring mitochondria is very small.

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