Oxygen diffusion-concentration product in rhodopsin as observed by a pulse ESR spin labeling method

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ABSTRACT Permeation of molecular oxygen in rhodopsin, an integral membrane protein, has been investigated by monitoring the bimolecular collision rate between molecular oxygen and the nitroxide spin label using a pulse electron spin resonance (ESR) T_1 method. Rhodopsin was labeled by regeneration with the spin-labeled 9-*cis* retinal analogue in which the β-ionone ring of retinal is replaced by the nitroxide tetramethyl-oxypyrrolidine ring. The bimolecular collision rate was evaluated in terms of an experimental parameter $W(x)$, defined as $T_1^{-1}(\text{air}, x) - T_1^{-1}(N_2, x)$ where T_1's are the spin-lattice relaxation times of the nitroxide in samples equilibrated with atmospheric air and nitrogen respectively, which is proportional to the product of local oxygen concentration and local diffusion coefficient (transport). W-values at the β-ionone binding site in spin-labeled rhodopsin are in the range of 0.02–0.13 μs^{-1}, which are 10–60 times smaller than W's in water and 1.1–20 times smaller than in model membranes in the gel phase, indicating that membrane proteins create significant permeation resistance to transport of molecular oxygen inside and across the membrane. W (thereby the oxygen diffusion-concentration product) is larger in the meta II-enriched sample than in rhodopsin, indicating light-induced conformational changes of opsin around the β-ionone binding site. W decreases with increase of temperature for both rhodopsin and meta II-enriched samples, suggesting that temperature-induced conformational changes take place in both samples. These changes were not observable using conventional ESR spectroscopy. It is concluded that W is a sensitive monitor of conformational changes of proteins.

INTRODUCTION

Intensive effort has been made in many laboratories to determine the accessibility and penetration of small molecules to specific sites in proteins. The mechanism by which small molecules reach various domains in proteins is of fundamental interest in the study of protein dynamics and enzyme mechanisms (Case and Karplus, 1979; Perutz, 1989). Accessibility and penetration have been studied by observing hydrogen exchange behavior (Englander and Kallenbach, 1983), fluorescence and phosphorescence quenching of tryptophan (Lakowicz and Weber, 1973*a*, *b*; Eftink and Ghiron, 1981; Strambini et al., 1987; Calhoun et al., 1988), and enhanced relaxation of spin labels attached to a specific residue of a protein (Altenbach et al., 1989, 1990).

One of the most extensively utilized molecules in these studies is molecular oxygen (Lakowicz and Weber, 1973*a*, *b*; Calhoun et al., 1983, 1988; Strambini et al., 1987; Altenbach et al., 1989, 1990; Brunet et al., 1990). Molecular oxygen is an ideal choice for the probe because it has finite solubility in most domains in the protein interior (Lakowicz and Weber, 1973*b*; Calhoun et al., 1988) due to its small size and appropriate level of hydrophobicity. In addition, molecular oxygen can be easily added and removed by changing the partial pressure in the equilibrating gas mixture. Oxygen accessibility to various positions in the protein can be determined by measuring the extent of fluorescence and phosphorescence quenching of tryptophan residues or by observing oxygen-enhanced paramagnetic relaxation of spin labels.

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Previously, we have shown that molecular oxygen is a useful probe for examination of dynamics of phospholipid alkyl chains and structural conformability of neighboring lipids in model membranes (Subczynski and Hyde, 1981; Kusumi et al., 1982; Subczynski et al., 1989, 1991). Our method is based on measurement of the bimolecular collision rate between molecular oxygen and nitroxide spin labels attached to the lipid. It requires determination of the spin-lattice relaxation time (T_1) of the nitroxide in the presence and absence of oxygen, for which pulse saturation-recovery ESR techniques were used. An "oxygen transport parameter" $W(x)$ was introduced as a convenient quantitative measure of the collision rate between the spin label and molecular oxygen (Kusumi et al., 1982):

$$W(x) = T_1^{-1}(\text{air}, x) - T_1^{-1}(N_2, x). \quad (1)$$

Note that $W(x)$ is normalized to the sample equilibrated with atmospheric air. $W(x)$ is proportional to the product of the local concentration $C(x)$ and the local translational diffusion coefficient $D(x)$ of oxygen (thus, called a transport parameter),

$$W(x) = 8\pi p r_0 \cdot D(x) \cdot C(x), \quad (2)$$

where r_0 is the interaction distance between oxygen and the nitroxide radical spin labels (≈4.5 Å) and p is the probability that an observable event occurs when a collision does occur (Subczynski and Hyde, 1984; Hyde and Subczynski, 1984). It was concluded that the oxygen transport parameter is a useful monitor of membrane

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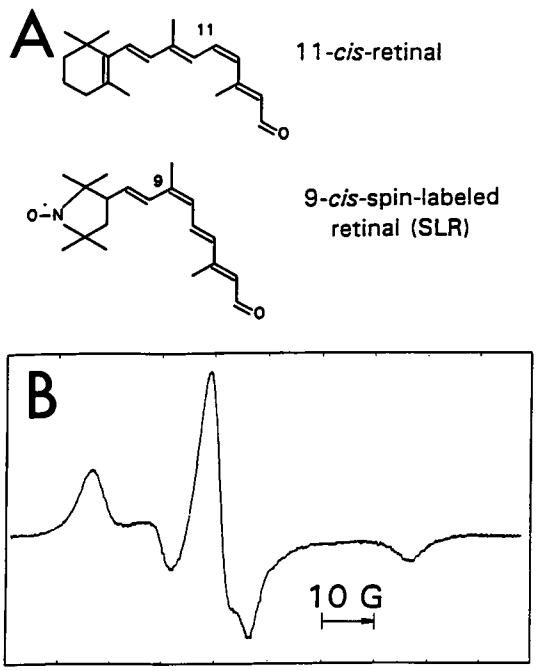

FIGURE 1 (*A*) Structures of 11-*cis* retinal and 9-*cis* spin-labeled retinal analog (SLR). (*B*) Conventional *X*-band ESR spectrum of SLR-rhodopsin recorded at 20°C. The maximum splitting value is 61.2 ± 0.5 G. The sample was deoxygenated. The incident microwave power was 1 mW and the 100 kHz field modulation width was 1 G.

fluidity that reports on translational diffusion of small molecules in the membrane (Kusumi et al., 1982).

The rationale for use of the spin-label T_1 method in these studies compared with other spectroscopic techniques lies in the fact that the time scale of T_1 (0.5–10 μs) is in the correct range to study many biological processes of interest. Nanosecond fluorescence lifetimes are in general too short to detect small changes in the collision rate under physiological pressures. The measurement of phosphorescence, which is in the millisecond range, is too sensitive to oxygen to be practical; if measurable phosphorescence intensities are to be obtained, extremely low concentrations of molecular oxygen (0.005% partial pressure) must be used, which in turn makes control of experiments difficult. An additional advantage of the spin-label T_1 method is that spin probes are smaller with locations that are better defined.

In the present research, we report extension of the method to an integral membrane protein. Bovine rhodopsin, the visual pigment in the rod outer segment (ROS) membrane, can be specifically labeled by removal of the native chromophore and replacement with 9-*cis* spin labeled retinal (SLR, the structure shown in Fig. 1 *A;* Renk et al., 1987, 1988). In this probe, the natural 11-*cis* double bond is replaced with a 9-*cis* bond, which is well known not to affect pigment formation or properties significantly (for reviews, see Balogh-Nair and Nakanishi, 1982; Crouch, 1986), and the β-ionone ring is replaced with a tetramethyl-oxypyrrolidine ring. The ring methyl groups were shown to be essential for filling of the binding site and for pigment formation (Lacy et al., 1982; Crouch and Or, 1983). It was proposed that hydrophobic binding of the β-ionone ring to opsin, the apoprotein, is the rate limiting step in the regeneration process of rhodopsin (Matsumoto and Yoshizawa, 1975; Matsumoto et al., 1980). The cross-linking study using a photoactivatable retinal analog indicated that the β-ionone ring orients toward helices *C* and *F* (Nakayama and Khorana, 1990). Because the location of the nitroxide is very close to the cross-linker group in this retinal analogue, the site we monitor with the SLR is proposed to be near both *C* and *F* helices and is critically important for regeneration of rhodopsin.

The oxygen transport parameter (thereby the oxygen diffusion-concentration product) at this location was measured in the rhodopsin state and under conditions that favor the stable formation of metarhodopsin (meta) II state. Meta II is an important intermediate in visual reception because it induces activation of phosphodiesterase, the first step of signal amplification. The oxygen transport parameter was found to be increased in the meta II state. In addition, anomalous temperature-dependence of *W*, decrease of *W* with increase of temperature, was observed, suggesting temperature dependent conformational changes of rhodopsin and meta II in the range of 0 and 20°C.

MATERIALS AND METHODS

All experiments were carried out under dim red light or in the dark unless specified. Bovine ROS membranes were isolated from frozen retinae (Hormel, Austin, MN) as previously described (Papermaster and Dreyer, 1974). Synthesis and characterization of SLR and formation and characterization of SLR pigment (λ_{max} = 460 nm) have been reported (Renk et al., 1987, 1988). The SLR-rhodopsin was suspended in 67 mM K_2HPO_4/KH_2PO_4 buffer in the dark. For generation of the sample that contains metä II as the major species, SLR-rhodopsin in ROS membranes was resuspended in 10 mM sodium acetate/acetic acid at pH 4.5 containing 65 mM KCl, 0.2 mM $CaCl_2$, and 0.2 mM $MgCl_2$ and irradiated with white light from a slide projector (model 4400, Eastman Kodak Co., Rochester, NY) at 0°C for 90 s with stirring and then incubated at 22°C for 15 min in the dark (Renk et al., 1987, 1988). Under these conditions, pararhodopsin may also be generated in a smaller amount (Matthews et al., 1963; Yoshizawa and Shichida, 1982; Renk et al., 1987).

The L-α-dimyristoylphosphatidylcholine and L-α-dioleoylphosphatidylcholine membranes used in this work were multilamellar dispersions of phospholipids containing 1 mol% of 16-doxyl stearic acid spin labels and were prepared as described (Subczynski et al., 1991).

For ESR studies, the membrane suspension was centrifuged at 8,000 *g* for 5 min, and the membranes in the loose pellet were drawn into a gas-permeable capillary (0.5 mm, i.d.) made of methylpentene polymer, TPX (Hyde and Subczynski, 1989). This plastic is permeable to nitrogen, oxygen and carbon dioxide and is substantially impermeable to water. The concentration of oxygen in the sample was controlled by equilibrating the sample with the same gas that was used for temperature control, i.e., a controlled mixture of nitrogen and dry air adjusted with flowmeters (model 7631H-604, Matheson Gas Products, Secaucus, NJ; Kusumi et al., 1982; Hyde and Subczynski, 1989).

T_1's of the spin labels were measured at *X*-band using the long-pulse saturation-recovery technique (Kusumi et al., 1982; Subczynski et al.,

1989, 1991). With long and intense microwave pulses, the spin system approaches a steady state at which the population of spins at each energy level tend to be equalized. After the saturating pulse is turned off, the recovery of the spin system to Boltzmann equilibrium is monitored with a weak observing power. In general, the nitrogen nuclear spin-lattice relaxation time is much shorter than the electron spin-lattice relaxation time for spin labels in membranes (Yin et al., 1987) and single exponential decays are found. The duration of the saturating pulse is then not critical. However, if the electron spin-lattice relaxation becomes sufficiently short by introduction of high concentrations of molecular oxygen, multiple exponential signals are expected and the long-pulse techniques should be used. The saturation-recovery spectrometer is based on the design of Huisjen and Hyde (1974) and is interfaced with a loop-gap resonator. A field-effect transistor microwave amplifier has been recently introduced. Typically, 2×10^7 decay curves were accumulated with 128–512 data points on each decay. The accumulation time was typically 5 min. The apparatus used here was described previously (Yin et al., 1987; Subczynski et al., 1991). Conventional ESR spectra were obtained with a Varian E-109 X-band spectrometer.

RESULTS AND DISCUSSION

The conventional ESR spectrum of the SLR pigment measured in the dark is shown in Fig. 1 *B*. The overall shape of the spectrum indicates that the nitroxide motion is strongly suppressed. No weakly immobilized component is present, and, therefore, we assume that only one species of pigment in one environment is present. Under the conditions in which meta II rhodopsin was enriched by irradiation at pH 4.5 (see Materials and Methods), the ESR spectrum was slightly affected, but indicated the presence of a single spectral component.

The spin-lattice relaxation time was measured at several temperatures not only in rhodopsin itself, but also under conditions that favor formation of the meta II state. The samples were equilibrated either with nitrogen gas or atmospheric air. Typical saturation-recovery curves are shown in Fig. 2 *A*. All measurements of T_1's were made on the central line ($M_I = 0$). The recovery followed a single exponential curve under all experimental conditions and instrumental settings used in this work. The accuracy of T_1 measurement was better than 5%.

Spin-lattice relaxation times for rhodopsin (*solid symbols*) and the meta II-enriched (*open symbols*) sample are plotted as a function of temperature in Fig. 2 *B*. In the absence of oxygen (*circles*), T_1's are long and typical of those for slowly tumbling spin labels (Huisjen and Hyde, 1974; Kusumi et al., 1982). T_1 values in the absence of oxygen decrease as the temperature is raised, reflecting enhanced motion. T_1 for the meta II-enriched sample is longer than that for the rhodopsin sample at 0°C, indicating conformational changes in the region of the β-ionone binding site. This difference was not detected at 20°C.

In the presence of atmospheric oxygen (Fig. 2 *B, triangles*), T_1 values are decreased. In addition, a surprising temperature dependence of T_1 was observed. In both rhodopsin samples and meta II-enriched samples, T_1 increases with increase of temperature. Because T_1 in the absence of oxygen decreases as the temperature is raised, this finding must indicate decreased oxygen transport around the β-ionone ring binding site at higher temperature.

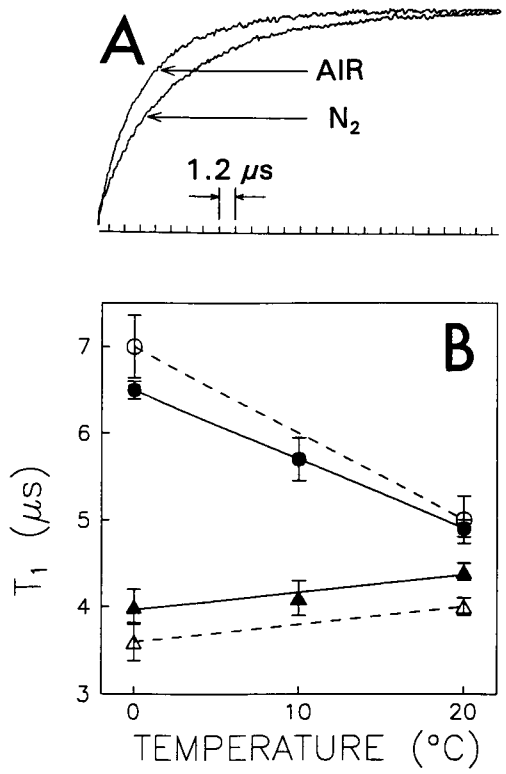

FIGURE 2 (*A*) Typical saturation-recovery signals of SLR-rhodopsin measured at 0°C for samples saturated with air and with nitrogen gas. Experimental conditions: pump power = 60 mW (microwave field = 1.2 G in the rotating frame), observing power = 0.2 mW, accumulation of 2^{13} decays during 5.5 min with 256 data points/decay. T_1 is 4.0 and 6.4 μs (pump width = 10 and 15 μs) for samples equilibrated with air and nitrogen gas, respectively. (*B*) T_1 values for SLR-rhodopsin (●, ▲) and meta II-enriched SLR-rhodopsin (○, △) plotted as a function of temperature. The samples were equilibrated with nitrogen (●, ○) and with air (▲, △). Equilibration was carried out at the same temperature as for the pulse ESR measurement. Average of five measurements for two independent preparations. The bars indicate standard deviations. The temperature dependent changes for both samples are statistically significant ($P < 0.05$ using Student's *t* test). The temperature was cycled to check the reproducibility and hysterisis.

The oxygen transport parameters were calculated from the values shown in Fig. 2 on the basis of Eq. 1 and are summarized in Table 1. The oxygen transport parameter W indeed decreases with increase in temperature for both rhodopsin and the meta II-enriched sample. This result indicates that temperature-induced conformational changes of the pigment occur between 0 and 20°C in both rhodopsin and the meta II state.

W values determined at the location of the nitroxide are small. They are smaller by a factor of 1.1–20 compared with W in the gel phase of L-α-dimyristoylphosphatidylcholine membranes (see Table 1), and are

TABLE 1 *W* values (μs^{-1}) for SLR-rhodopsin and meta II-enriched SLR-rhodopsin compared with those in model membranes in the gel and fluid phases*

Temperature	SLR-rhodopsin	Meta II-enriched	Gel-phase membrane‡	Fluid-phase membrane‡
°C				
0	0.096 (±0.012)	0.130 (±0.020)	0.14	1.16
10	0.070 (±0.020)	N.D.§	0.25	1.59
20	0.020 (±0.008)	0.050 (±0.012)	0.39	2.12

* *W* in water is 1.3 μs^{-1}. The temperature dependence of *W* in water is negligible due to the opposite temperature dependences of the diffusion coefficient and concentration of molecular oxygen in water (Subczynski et al., 1989). ‡Determined by using 16-doxyl stearic acid spin labels in L-α-dimyristoylphosphatidylcholine (gel phase) and L-α-dioleoylphosphatidylcholine (fluid phase) membranes. §N.D. = not determined. The difference between the rhodopsin and the meta-II enriched samples, and that between 0 and 20°C for both samples are statistically significant ($P < 0.05$ using Student's *t* test).

smaller by a factor of 10–60 compared with *W* in water (1.3 μs^{-1}). *W* in rhodopsin is smaller by a factor of 15–100 compared with that in the liquid-crystalline phase of L-α-dioleoylphosphatidylcholine membranes. The solubility of dioxygen in water at room temperature is ≈0.3 mM (Hitchman, 1978) and may be ≈5 times higher in hydrophobic environments (Linke, 1965; Battino et al., 1968). The results obtained in this work thereby indicate that membrane proteins present significant permeation resistance to molecular oxygen inside and across the membrane (Subczynski et al., 1989, 1991).

These data indicate that the β-ionone binding site is protected from the penetration of small molecules, even as small as dioxygen. This conclusion from *W* data is consistent with data by conventional ESR spectroscopy that indicated that the label environment is protected from water penetration and is tightly packed (Renk et al., 1987), and with an electron double resonance study that showed no interaction between SLR and spin-labeled lipid in the membrane (Renk et al., 1988).

Matsumoto et al. (1980) previously studied the kinetics of binding of various retinal analogues and fragments to opsin, and proposed that the β-ionone binding site is isolated from water and that β-ionone binding is the rate-limiting (slow) step for the binding of retinal to opsin. Our results are consistent with their model.

W values are larger for the meta II-enriched sample than for the rhodopsin sample at both 0 and 20°C. This result suggests that light-induced conformational changes take place around the binding site of the β-ionone ring near *C* and *F* helices in the meta II state. It is concluded that *W* is a sensitive monitor of conformational changes of proteins.

In samples equilibrated with atmospheric air, T_1 values are smaller by 40–50% at 0°C and by 20–30% at 20°C (Fig. 2 *B*) compared with those in the absence of oxygen; the time scale for the bimolecular collision rate with oxygen is comparable to the spin-label T_1's under the physiological conditions. The good match of the time scales of the spin-label T_1's and the collision rate of molecular oxygen under atmospheric conditions makes the spin-label T_1 method particularly appropriate for oxygen permeation studies.

In a series of studies, we have investigated oxygen transport in model membranes. It was shown that the oxygen transport parameter is a sensitive monitor for packing defects and dynamics of lipids in membranes (Subczynski et al., 1991). In the present paper, we have shown that the oxygen transport parameter can be used to address problems of conformational changes and dynamics of membrane proteins, and that it can be extended to studies of reconstituted membranes and biological membranes.

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