# Molecular Organization and Dynamics in Bacteriorhodopsin-Rich Reconstituted Membranes: Discrimination of Lipid Environments by the Oxygen Transport Parameter Using a Pulse ESR Spin-Labeling Technique†

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ABSTRACT: Molecular organization and dynamics in protein-rich membranes have been studied by investigating transport (diffusion-concentraton product) of molecular oxygen at various locations in reconstituted membranes of bacteriorhodopsin (BR) and L-α-dimyristoylphosphatidylcholine. Oxygen transport was evaluated by monitoring the bimolecular collision of molecular oxygen with four types of nitroxide lipid spin labels placed at various locations in the membrane. The collision rate was estimated from the spin–lattice relaxation times ($T_1$'s) measured at various oxygen partial pressures by analyzing the short-pulse saturation recovery ESR signals. CD spectra and decay of polarized flash-induced photodichroism of bacteriorhodopsin indicated that BR molecules are monomers in reconstituted membranes with a lipid/BR molar ratio of 80 (80-rec) and are 25% monomers and 75% trimers plus oligomers of trimers when the lipid/BR ratio is 40 (40-rec). In the 80-rec, the lipid environment is homogeneous on a microsecond scale ($T_1$), probably because the exchange rate of lipids between the bulk and the boundary regions is greater than the $T_1$ relaxation rate ($\approx 10^6$ s$^{-1}$). The oxygen collision rate in the hydrophobic region of the 80-rec membrane is smaller by a factor of 1.6 than in that of the lipid membrane without BR, and the effect of BR in decreasing the collision rate is independent of the "depth" in the hydrophobic region. In the 40-rec, two collision rates were observed, one of which is close to those for purple membrane (or the gel-phase membrane), while the other is about the same as was measured in the 80-rec. These results indicate the presence of a special lipid environment (slow oxygen-transport domain, i.e., likely to be self-associated BR domain) in the 40-rec in which oxygen transport is smaller by a factor of 5 than in the bulk plus boundary region. The residence time of lipids in the slow oxygen-transport domain is longer than $10^{-6}$ s ($T_1$). It is speculated that the slow oxygen-transport domain consists of lipids in contact with two proteins and/or lipids in contact with protein and boundary lipids. It follows that alkyl chains and BR are closely packed in the slow oxygen-transport domain, with few vacant pockets to allow entrance and movements of even small molecules such as molecular oxygen. It is concluded that molecular oxygen makes a particularly useful probe for studies of molecular organization in protein-rich membranes.

Recently, a method has been developed in which molecular oxygen is used as a probe to study the three-dimensional molecular organization and dynamics in membranes (Kusumi et al., 1982a; Subczynski et al., 1989, 1991a). The rates of bimolecular collisions between oxygen and nitroxide spin labels placed at specific locations in the membrane have been evaluated from the spin–lattice relaxation times ($T_1$'s)[1] of spin labels using a pulse ESR technique. Molecular oxygen has a unique characteristic as a membrane probe; it can enter the small vacant pockets that are transiently formed in the membrane because of its small size and appropriate level of hydrophobicity. Therefore, the bimolecular collision rate is sensitive to the dynamics of gauche–trans isomerization of lipid alkyl chains and to the structural nonconformability of neighboring lipids (Pace & Chan, 1982; Subczynski et al., 1991a). Using molecular oxygen as a probe, several unusual effects of cholesterol and alkyl chain unsaturation were found in phosphatidylcholine (PC) membranes: in the absence of cholesterol, incorporation of either a cis or trans double bond in the alkyl chain *decreases* the oxygen collision rate at all locations in the membrane, and incorporation of cholesterol in unsaturated membranes *increases* the collision rate in the middle of the bilayer (Subczynski et al., 1989, 1991a).

We now report the extension of this method to the investigation of reconstituted membranes, with special at-

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[1] Abbreviations: BR, bacteriorhodopsin; DMPC, L-α-dimyristoylphosphatidylcholine; PC, phosphatidylcholine; 40- and 80-rec, reconstituted membranes of DMPC/BR ratios of 40 and 80, respectively; L/P, lipid-to-protein ratio in mol/mol; SASL, stearic acid spin label; 5- and 16-SASL, 5- and 16-doxylstearic acid spin labels, respectively; 12-PC, 1-palmitoyl-2-(12-doxylstearoyl)phosphatidylcholine; Tempo–PC, tempocholine–dipalmitoylphosphatidic acid ester; $T_1$, spin–lattice relaxation time.

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tention paid to membranes crowded with integral membrane proteins. Molecular motion in protein-rich membranes has been a subject of intensive study in recent years (Ryan et al., 1988; Chazotte & Hackenbrock, 1988; Abney et al., 1989; Minton, 1989; Saxton & Owicki, 1989; Edidin & Stroynowski, 1991; Saxton, 1992) because many biological membranes contain functional domains that are rich in integral membrane proteins (Kusumi & Hyde, 1982; Edidin, 1990, and papers cited therein). For membranes and membrane domains (protein-rich domains) that possess very small amounts of bulk lipids, we have a very limited understanding of the molecular organization and dynamics. The basic concept of the fluid-mosaic model (Singer & Nicholson, 1972), an essential point of which is that the membrane proteins are floating in a sea of excess lipid molecules, is not applicable or may even be misleading in understanding the diffusion-solubility characteristics of solutes in a membrane in which most lipid molecules are in contact with one or two protein molecules and protein association is a common occurrence.

In the present research, we have studied the behavior of molecular oxygen in protein-rich reconstituted membranes to gain insight into the organization and the conformational dynamics of the boundary lipids around a membrane protein and of the trapped lipids between proteins. We are particularly concerned with the molecular packing and the dynamics at the interface between lipids and integral membrane proteins in protein-rich domains. Bacteriorhodopsin (BR) was selected as a membrane protein because the self-association states can be manipulated by varying the lipid/BR ratio. To our knowledge, no studies of the three-dimensional translational diffusion of small solutes in reconstituted membranes have been reported so far.

To monitor the local diffusion-solubility characteristics of oxygen molecules in the membrane, the bimolecular collision rate between oxygen (a fast relaxing species) and the nitroxide spin label (a slow relaxing species) placed at specific locations in the membrane has been evaluated in terms of an oxygen-transport[2] parameter [$W(x)$] using a pulse ESR spin-labeling method (Kusumi et al., 1982a; Subczynski et al., 1989, 1991a). $W(x)$ is defined as

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

where the $T_1$'s are the spin–lattice relaxation times of the nitroxide in samples equilbrated with atmospheric air and nitrogen, respectively. $W(x)$ is proportional to the product of the local concentration $C(x)$ and the local translational diffusion coefficient $D(x)$ of oxygen (thus called "transport" parameter) at a "depth" $x$ in a membrane that is in equilibrium with atmospheric air:

$$W(x) = AD(x)C(x), A = 8\pi pr_0 \quad (2)$$

where $r_0$ is the interaction distance between oxygen and the nitroxide radical spin labels (≈4.5 Å; Molin et al., 1980) and $p$ is the probability that an observable event occurs when a collision does occur[3] and is very close to 1 (Hyde & Subczynski, 1989).

Our previous data on $W$ in vertebrate rhodopsin, which belongs to the same class of integral membrane protein with seven transmembrane helices, showed that $W$ in the protein (monitored at the binding site of the β-ionone of retinal) is small; it is smaller by a factor of 10–60 compared with $W$ in water and by factors of 1.1–20, and 15–100 compared with $W$ in L-α-dimyristoylphosphatidylcholine (DMPC) membranes in the gel and liquid-crystalline phases, respectively (Subczynski et al., 1992b). In the present study, we have examined the oxygen-transport parameter $W(x)$ in the lipid region in reconstituted membranes as a function of the lipid-to-protein ratio (described in mole/mole in this paper, L/P), temperature, and location of spin labels in the membrane.

## EXPERIMENTAL PROCEDURES

*Materials.* DMPC was obtained from Sigma, 5- and 16-doxylstearic acid spin labels (5- and 16-SASL) were from Molecular Probes (Junction City, OR), and 1-palmitoyl-(12-doxylstearoyl)phosphatidylcholine (12-PC) was from Avanti Polar Chemicals (Alabaster, AL). Tempocholine–dipalmitoylphosphatidic acid ester (Tempo–PC) was a generous gift from Dr. S. Ohnishi (Kyoto University, Kyoto, Japan).

*Preparation of BR–DMPC Reconstituted Membranes.* Purple membrane was isolated from *Halobacterium halobium* JW3 according to the established procedure (Oesterhelt & Stoeckenius, 1974). It was solubilized with 1% Triton X-100 and reconstituted with added DMPC by removing the detergent by extensive dialysis against 0.1 M sodium acetate buffer at pH 5.0 (15 changes of 200-times excess dialysis buffer for 10 days; Cherry et al., 1978). Residual detergent as determined by radiolabeled Triton was less than 0.5 mol % of total lipid in reconstituted membrane suspension. The reconstituted membranes were purified by sucrose linear gradient centrifugation (5–45% w/w). Only a single band, the density of which varies with the L/P, was visible for each preparation, which was then recovered for further experiments. The L/P ratio was determined on the basis of phosphorus (Bartlett, 1959) and protein measurements (Lowry et al., 1951). For spin labeling with SASL, the concentrated labels in ethanol (1 mM) were added to the reconstituted membranes (final ethanol concentration below 0.5% v/v). For experiments with the PC labels, the membranes were reconstituted with the labels. The final spin label concentrations were between 0.7 and 1.2 mol % of the total lipid. Purple membrane was labeled with the PC spin labels by solubilizing both the membrane and the spin labels in a Triton X-100 solution followed by dialysis (L/P decreased from ≈12 of purple membrane to ≈9 of the labeled membrane).

*Measurements of the CD Spectra and the Decay of the Polarized Flash-Induced Photodichrosim of Retinal.* CD spectra for the reconstituted membranes were obtained in 0.1 M sodium citrate at pH 5.0 with a Jasco J-500 spectrometer (Tachikawa, Japan). The CD spectra for the reconstituted membranes were obtained by subtracting the spectrum for bleached BR in reconstituted membranes that were prepared by irradiation in the presence of 0.1 M hydroxylamine (Oesterhelt & Schuhmann, 1974). The decay of the photoinduced dichroism of BR was measured as described previously (Kouyama et al., 1981).

*ESR Measurement.* Buffer used with the SASLs was 0.1 M sodium borate at pH 9.5. To ensure that all carboxyl groups of the SASLs were ionized in the PC membranes, a rather high pH was chosen (Sanson et al., 1976; Egret-Charlier et al., 1978; Kusumi et al., 1982a,b, 1986). For the measurements with Tempo–PC and 12-PC, 0.1 M sodium acetate buffer at

[2] In the present paper, the word "transport" is used in its basic physical sense, indicating the product of the (local) translational diffusion coefficient and the (local) concentration of oxygen in the membrane. Active transport across the membrane is not the subject of this paper.

[3] A is remarkably independent of the hydrophobicity and viscosity of the solvent and of the spin label species (Hyde & Subczynski, 1984; Subczynski & Hyde, 1984).

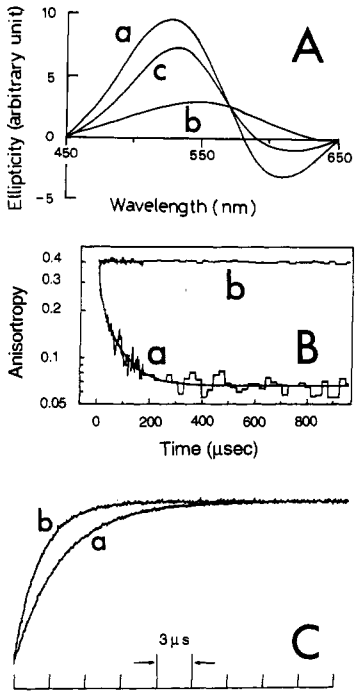


FIGURE 1: (A) CD spectra of BR–DMPC reconstituted membranes. These spectra were obtained by subtracting those for bleached BR in reconstituted membranes: (a) 80-rec at 4 °C; (b) 80-rec at 35 °C; (c) 40-rec at 35 °C. (B) Polarized flash-induced anisotropy decay of retinal in BR in the 40-rec (a) and in purple membrane (b) observed at 570 nm at 30 °C. (C) Typical saturation recovery signals for 12-PC in the 80-rec at 30 °C: (a) without oxygen; (b) in the presence of 10% air.

pH 5.0 was used to ensure stability of the sample. The results for these two samples were unchanged at pH 9.5. The reconstituted membranes were centrifuged briefly with an Airfuge (Beckman, Palo Alto, CA), and the loose pellet (≈20% lipid w/w) was used for ESR measurement. Dilution of the pellet did not cause any detectable changes in the results. The sample was placed in a capillary (i.d. = 0.5 mm) made of gas-permeable polymer, TPX (Hyde & Subczynski, 1989). The concentration of oxygen in the sample was controlled by equilibration with the same gas that was used for temperature control, i.e., a controlled mixture of nitrogen and dry air adjusted with flow meters (Matheson Gas Products Model 7631H-604; Kusumi et al., 1982a; Hyde & Subczynski, 1989). The $T_1$'s of the spin labels were determined by analyzing the saturation-recovery signal of the central line obtained by the short-pulse saturation-recovery ESR technique (Yin et al., 1987).

## RESULTS

*Self-Association States of BR in Reconstituted Membranes.* Four types of membrane preparations were used in this work: (1) purple membrane, (2) the reconstituted membrane with a L/P of 40 (40-rec), (3) the reconstituted membrane with a L/P of 80 (80-rec), and (4) the DMPC membrane, i.e., the membrane reconstituted without BR.

The self-association states of BR in the reconstituted membranes were studied by measuring the CD spectra of BR retinal (Heyn et al., 1975; Ebrey et al., 1977; Cherry et al., 1978) and by observing the decay of the polarized flash-induced photodichroism of retinal (Kouyama et al., 1981; Cherry & Godfrey, 1981). The CD spectra are shown in Figure 1A. The spectrum for the 80-rec shows no indication of exciton coupling of retinals, indicating that BR molecules are monomers, while that for the 40-rec shows evidence of exciton

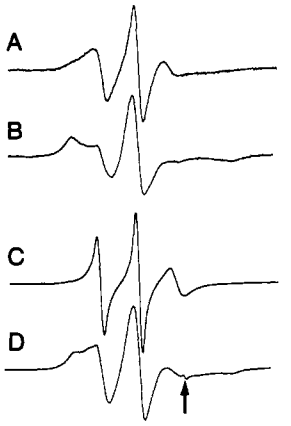


FIGURE 2: Typical conventional ESR spectra of (A) 12-PC in the 80-rec and (B) in purple membrane and (C) 16-SASL in the 80-rec and (D) in purple membrane obtained at 30 °C. The arrow in (D) indicates the most conspicuous part of the spectra that shows the presence of very small amounts of 16-SASL in the aqueous phase.

coupling. Comparison with spectra obtained at 4 °C (below the phase transition temperature), at which extensive aggregation of BR takes place, indicates that ≈75% of the BR molecules in the fluid phase of the 40-rec are trimers or larger aggregates of trimers (assuming that all BR molecules are aggregated below the phase transition temperature and that the CD spectra for timers and aggregates are the same. In addition, we assume that CD spectra can be interpreted by excitons (Cassim, 1992, and references therein).

To estimate the size of the BR aggregates in the 40-rec, the decay of polarized flash-induced photodichroism was measured (Figure 1B). Anisotropy decays rapidly to its limiting value (0.069) even in the 40-rec, showing the absence of large aggregates (Cherry & Godfrey, 1981). The time scale of the decaying component is shorter than 100 μs, and no component with longer decay times was observed. These results suggest that the sizes of the diffusing units of BR in the 40-rec are monomers, trimers, and oligomers of trimers (Cherry & Godfrey, 1981; Peters & Cherry, 1982).[4] In the case of purple membrane, no rotational diffusion of BR was detected [$r(t)$ = 0.39] on the time scale of 3 ms. In summary, BR in the 80-rec are monomers, BR in the 40-rec are 25% monomers and 75% trimers (plus oligomers of trimers), and BR in purple membrane are totally aggregated. These results are in agreement with the data by freeze-fracture electron microscopy of BR-reconstituted membranes (Lewis & Engelman, 1983; Pearson et al., 1983). Our results are also consistent with the data of Sternberg et al. (1992), who showed that trimers of BR in reconstituted membranes form hexagonal 2D arrays similar to that found in native purple membrane only in the presence of *both* native purple membrane lipids and 4 M NaCl. The reconstituted membranes used here should contain native lipids, but the salt concentration used was 0.1 M.

*Measurement of $T_1$ in Reconstituted Membranes.* Conventional ESR spectra of 12-PC and 16-SASL in the 80-rec and purple membrane are shown in Figure 2. The spectra suggest the presence of at least two lipid environments in the time scale of the magnetic anisotropy of $10^{-8}$ s. Probability

[4] The low signal-to-noise ratio of the decay curves prevented us from carrying out a detailed analysis to detect the small changes in the rotational diffusion rate that may be induced by transient association of BR (Kusumi & Hyde, 1982). Saturation transfer ESR spectroscopy was not performed because the spin labels could not be strongly immobilized on the protein (nor has any report been published on saturation transfer spectroscopy of spin-labeled BR).

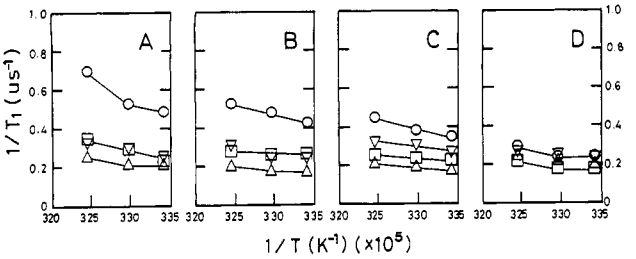


FIGURE 3: $1/T_1$ vs $1/T$ for Tempo–PC (▽), 5-SASL (△), 12-PC (□), and 16-SASL (○) in various membranes in the absence of oxygen: (A) DMPC membrane; (B) 80-rec; (C) 40-rec; (D) purple membrane. Accuracy of estimating $T_1$ is within ±5%.

of concentration of the spin labels in particular domains is low because no apparent spin exchange is seen in these spectra. Small amounts of 16-SASL are present in the aqueous phase in purple membrane samples, but its effect on the estimate of $T_1$ is negligible because the signal level of this component is small (Figure 2D).

The buffer used with the SASLs was 0.1 M sodium borate at pH 9.5 throughout this study. This was to ensure that all carboxyl groups of the SASLs were ionized in the PC membranes (Sanson et al., 1976; Egret-Charlier et al., 1978; Kusumi et al., 1982a,b, 1986). For the measurements with Tempo–PC and 12-PC, 0.1 M sodium acetate buffer at pH 5.0 was used to ensure stability of the sample. Since the results for these two samples (Tempo–PC and 12-PC) were unchanged at pH 9.5, data obtained at pH 5.0 are shown in this paper.

Typical saturation-recovery curves for 12-PC in the presence and absence of air are displayed in Figure 1C. All recovery curves obtained in this work can be analyzed as either single or double exponentials. The decay time constants were determined with an accuracy of ±5%. Saturation-recovery measurements were carried out systematically as a function of the partial pressure of oxygen in the equilibrating gas mixture, temperature (26, 30, 35 °C), L/P, and location of the spin labels in the membrane (the headgroup region with Tempo–PC, the hydrophobic region near the membrane surface with 5-SASL, the inner regions of the membrane with 12-PC, and the central part of the bilayer with 16-SASL).

In the absence of oxygen, all recovery curves could be fitted to a single-exponential function, while the conventional ESR spectra of 12-PC in the 40- and 80-recs indicated the presence of at least two lipid environments (see following sections for discussion). Overall variation in $T_1$ for the four spin labels in the four types of membranes used in this work in the absence of oxygen is only a factor of 3, in the range 1.5–5 μs (Figure 3).

*Oxygen-Transport Parameter in BR-Reconstituted Membranes.* In Figure 4, $1/T_1$ for 12-PC in four different membranes at 30 °C is shown as a function of oxygen concentration in the equilibrating gas mixture. In DMPC, purple, and 80-rec membranes, all decay curves (not only for 12-PC but for all spin labels used) could be fitted to a single-exponential function at the oxygen concentration used in this work, indicating that the lipid environment is homogeneous in terms of oxygen transport in the microsecond range ($T_1$) in these membranes. Specifically, we found one-component recovery (both in the presence and in the absence of oxygen) even in the 80-rec, in which two lipid environments, the boundary and the bulk regions, are present. This is probably because the lipid-exchange rate between the bulk and the boundary regions ($\approx 10^7$ s$^{-1}$; East et al., 1985; Ryba et al., 1987) is greater than the $T_1$ relaxation rate.

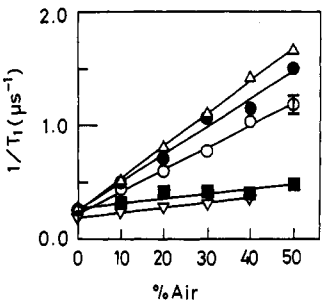


FIGURE 4: Plot of $1/T_1$ for 12-PC in various reconstituted membranes at 30 °C as a function of the molar ratio of air in the equilibrating gas mixture. DMPC membranes without BR (△), 80-rec (○), 40-rec (●, ■), and purple membrane (▽, reconstituted with the added spin label. See Preparation of BR–DMPC Reconstituted Membranes.)

All plots of $1/T_1$ for these membranes show a linear dependence on oxygen concentration between 0 and 50% air. The oxygen-transport parameter, $W(x)$, can be obtained from the slope of the linear plot. The presence of BR decreases $W(x)$ in the membrane.

In the 40-rec, in which BR molecules are crowded, two-exponential recovery signals were observed in the presence of oxygen with 12-PC (Figure 4). The two time constants are close to the $T_1$ values for 12-PC in purple membrane and in the 80-rec. Both components show linear relationships with oxygen concentration in the sample. When these data points are extrapolated to 0% air, both components reach the same value ($0.25 \times 10^6$ s$^{-1}$), which is the same as that determined in the absence of oxygen (Figure 4). These results can be explained by a model in which two lipid environments, possessing different oxygen-transport rates and a slow exchange rate of lipids between them, are present in the membrane; in this case, the saturation recovery signal is expected to be simply a double-exponential curve with time constants of $T_1^{-1}(R_{air}, x_I)$ and $T_1^{-1}(R_{air}, x_{II})$, where

$$T_1^{-1}(R_{air}, x_I) = W(x_I)R_{air} + T_1^{-1}(N_2, x_I) \quad (3)$$

$$T_1^{-1}(R_{air}, x_{II}) = W(x_{II})R_{air} + T_1^{-1}(N_2, x_{II}) \quad (4)$$

Subscripts I and II refer to the two lipid environments, and $R_{air}$ is the fraction of air in the equilibrating gas mixture. In the experiments with 12-PC presented in Figure 4, $T_1^{-1}(N_2, x_I) = T_1^{-1}(N_2, x_{II}) = 0.25 \times 10^6$ s$^{-1}$, $W(x_I) = 2.4 \times 10^6$ s$^{-1}$, and $W(x_{II}) = 0.45 \times 10^6$ s$^{-1}$ at 30 °C. These results thereby indicate the existence of a special lipid environment in the 40-rec in which $W(x_{II})$ is small and the exchange of lipids with those in the bulk (plus boundary) region is slower than $\approx 10^6$ s$^{-1}$ ($T_1^{-1}$). We refer to this domain (domain II) as the "slow oxygen-transport (SLOT) domain" in this paper. We speculate that the SLOT domains are the small membrane regions made of self-associated BR (trimers and oligomers of trimers of BR). In contrast, domain I, consisting of the bulk and boundary lipids, in which oxygen transport is fast, is called the "bulk–boundary domain".

*Membrane Profiles of the Oxygen-Transport Parameter.* Membrane profiles of $W(x)$ determined at four different depths in the membrane at three temperatures are shown in Figure 5. Each point was determined from the slope of a plot of $1/T_1$ vs oxygen concentration, as in Figure 4. All spin labels showed similar behavior in this plot. Determination of the slope was based on the measurements at five to seven oxygen concentrations (two to three decay measurements each), and the accuracy of $W(x)$ was estimated to be better than ±10%. The

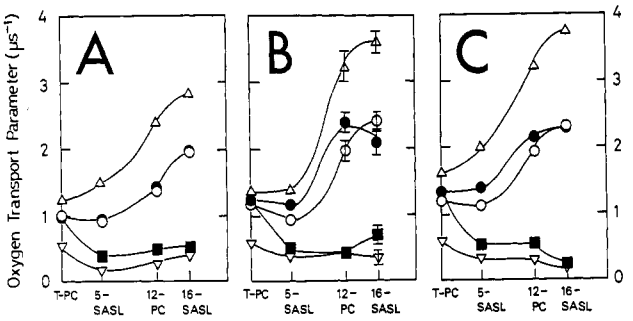


FIGURE 5: Profiles of the oxygen transport parameters across the membrane in the DMPC membrane without BR (△), 80-rec (○), 40-rec (●, ■), and purple membrane (▽, reconstituted only with the added spin label). The abscissa indicates the approximate depth in the membrane at which each spin label is placed (T-PC for Tempo–PC). (A) 26 °C; (B) 30 °C; (C) 35 °C.

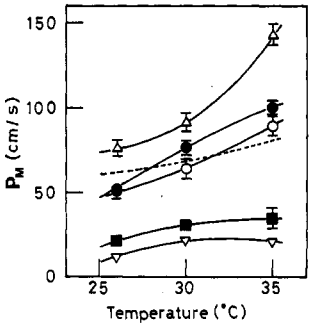


FIGURE 6: Oxygen permeability coefficients in the lipid domains in DMPC membrane without BR (△), 80-rec (○), 40-rec (●, ■), and purple membrane (▽) shown as a function of temperature. The broken line indicates the oxygen permeability coefficient of a water layer of the same thickness as the DMPC bilayer [data adopted from Subczynski et al. (1989)].

effect of BR on $W$(Tempo–PC), which monitors the interface region between the aqueous and the membranous phases, is smaller than that on $W$'s that monitor the hydrophobic region. In the following, only the effects in the hydrophobic region of the membrane are discussed.

In the 80-rec, $W(x)$ is smaller by a factor of 1.4–1.7 than in the DMPC membranes at all depths in the hydrophobic region.[5] The effect of BR on $W(x)$ has little dependence on the depth. This shows a marked contrast with the effect of cholesterol on $W(x)$, which is strongly depth-dependent (see below; Subczynski et al., 1989, 1991a).

In the 40-rec, two sets of membrane profiles of $W(x)$ were obtained (closed keys); one represents $W(x)$ in the bulk and boundary regions, and the other represents $W(x)$ in the SLOT domain, as discussed above. Since the SLOT domain was not observed in the 80-rec, it is proposed that the SLOT domain is the lipid region in which every lipid molecule is in contact with two proteins or with a protein and boundary lipids. To our knowledge, resolution of the bulk–boundary domain and the SLOT domain has been achieved for the first time. This has become possible by using molecular oxygen as a membrane probe, which we call "the method of discrimination by oxygen transport (DOT method)".

$W(x)$ in the hydrophobic region of the SLOT domain is not depth-dependent, i.e., the effect of BR in the SLOT domain is larger toward the central part of the membrane, as seen from the comparison with $W(x)$ profiles for DMPC membrane. In contrast, the effect of cholesterol in decreasing $W(x)$ is largest in and near the headgroup regions and is smallest in the central part of the bilayer. [In the presence of cholesterol, $W$(16-SASL) in unsaturated PC membranes actually increases (Subczynski et al., 1989a, 1991a).] The effect of incorporation of carotenoids, which have rigid conformations and span the membrane, is similar to that of BR (Subczynski et al., 1991b).

*Oxygen Permeability across the Membrane.* From the profiles of $W(x)$ shown in Figure 5, the oxygen permeability coefficient across the membrane ($P_M$) in each lipid domain was calculated (Figure 6) by the method of Subczynski et al. (1989, 1991a, 1992a). The presence of BR (1 BR molecule per 80 lipids) decreases $P_M$ by a factor of 1.5. $P_M$ in the SLOT domain and in the lipid portion of purple membrane is smaller by a factor of 3–8 compared with that in DMPC membranes.

[5] In comparable reconstituted membranes, Peters and Cherry (1982) showed that the lateral diffusion coefficient of a fluorescently labeled lipid is smaller by a factor of 3–4. This difference may be due to the differences between three-dimensional and two-dimensional diffusion or to a size-dependent diffusion mechanism.

## DISCUSSION

One of the fundamental questions in membrane biology is the mechanism by which the diffusion of proteins, lipids, and solutes is decreased in the presence of integral membrane proteins. Toward the understanding of the mechanism, in the present study, the L/P ratio was varied [80 and 40 for reconstituted membranes and 12 for purple membrane, the areal fractions of BR of the membrane cross section being $\approx 1/3, 1/2, 3/4$, respectively (Finean & Michell, 1981)]. In the 80-rec, in which BR molecules are mostly monomers, $W(x)$ is smaller by a factor of $\approx 1.6$ at all depths in the hydrophobic region compared with $W(x)$ in DMPC membrane. Two mechanisms may be working in parallel for the decrease of $W(x)$ in the presence of BR: (1) the decrease in the gauche–trans isomerization of the alkyl chain in the presence of BR (Subczynski et al., 1991a), that in the boundary region in particular (Almeida et al., 1992); and (2) the blocking of the diffusion pathway of molecular oxygen simply due to the presence of BR, in which $W$ is small (hard-core repulsion model; Pink, 1985; Saxton, 1987, 1992). Since $W$ in vertebrate rhodopsin is smaller by a factor of 15–100 compared with $W$ in DMPC membranes in the fluid phase (Subczynski et al., 1992b) and since the effect of BR in the 80-rec has no depth dependence in the hydrophobic region of the membrane, the decrease of $W(x)$ *in the 80-rec* may be largely explained by the hard-core repulsion model.

Many biological membranes and membrane domains are crowded with integral membrane proteins (Edidin, 1990). Nevertheless, little information on molecular organization and dynamics in protein-rich membranes is available. In this study, we have shown the presence of a lipid region (SLOT domain), which appears only in the protein-rich membrane, has a slow lipid exchange rate with the bulk–boundary domain, and has a small $W(x)$. The SLOT domain is likely to be the self-associated BR domain. The rate of lipid exchange between the inside and the outside of the SLOT domain is smaller than $\approx 10^6\ s^{-1}$ (see Figure 4). $W$(16-SASL) in the SLOT domain is $0.2–0.7 \times 10^6\ s^{-1}$, comparable to that in the gel phase of DMPC membranes, indicating that molecular dynamics in a protein-rich domain can be suppressed to the level of the gel-phase membrane. This is not due to the formation of large aggregates of BR in the 40-rec, as shown by the measurement of the decay of the polarized flash-induced photodichroism of BR.

As shown previously (Subczynski et al., 1991a), $W$ is strongly dependent on the rate of gauche–trans isomerization of lipid alkyl chains. A model for the mechanism of oxygen transport in the membrane has been proposed in which molecular oxygen

enters transient small vacant pockets created by gauche–trans isomerization of alkyl chains or by the structural nonconformability of neighboring lipids (mismatch in surface topography of molecules when two molecules are placed side by side in the membrane), and oxygen molecules jump from one pocket to an adjacent one or move along with the movement of the pocket itself (Träuble, 1971; Pace & Chan, 1982; Subczynski et al., 1991a; Haines & Liebovitch, 1993). This mechanism for the diffusion of small molecules in the membrane is quite different from that for the lateral diffusion of proteins and lipids in the membrane (Pace & Chan, 1982). It follows that the alkyl chains and BR are well packed in the SLOT domain, with few vacant pockets to allow entrance and movements of even small molecules such as molecular oxygen and/or that gauche–trans isomerization in this domain is as slow as that in the gel-phase membrane.

Another aspect of the difference in oxygen transport between the bulk–boundary domain and the SLOT domain may be dimensionality. While diffusion of oxygen in the bulk–boundary domain is a three-dimensional one, that in the SLOT domain may be two-dimensional or even one-dimensional diffusion in the normal direction of the membrane.

A variety of theories have been developed on the effect of integral membrane proteins on diffusion in the membrane (Pearson et al., 1983; Pink, 1985; Pink et al., 1986; Saxton, 1987, 1992; Abney et al., 1989). In the present research, we have shown that the hard-core repulsion model may be applicable to lipid-rich membranes. However, in these theories, the effect of membrane proteins on lipid conformational dynamics, which we have shown to be particularly important in protein-rich membranes and in the SLOT domain, has been completely neglected. The present results clearly indicate that the SLOT domain should be taken into account in theoretical and experimental investigations of protein-rich membranes.

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