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PULSE EPR DETECTION OF MEMBRANE DOMAINS

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Molecular oxygen makes a particularly useful probe to study the organization and dynamics of membrane domains and particularly protein-rich raft domains. Our new method is based on variation of the local diffusion-concentration product of molecular oxygen in different membrane domains, thus is called the method of discrimination by oxygen transport (DOT method). Oxygen transport is evaluated by monitoring the bimolecular collision of molecular oxygen with different types of nitroxide lipid spin labels placed at various locations in the membrane. The collision rate is estimated from the spin-lattice relaxation times (T_1s), measured at various oxygen partial pressures, by analyzing the short pulse saturation recovery EPR signals. In general, T_1s are close in the absence of oxygen, and the presence of different types of lipid domains can often be clearly manifested only after introducing molecular oxygen into the sample.

INTRODUCTION

Many functions of the plasma membrane are directly linked to the formation of membrane subdomains, in which specific lipids and proteins are assembled to carry out specific functions (Edidin, 1990; Edidin & Stroyanovski, 1991; Kusumi & Sako, 1996; Simons & Ikonen, 1997; Mouritsen & Andersen, 1998). One example is detergent-insoluble glycosphingolipid domains, which have been the subject of intensive studies in recent years (Simons & Ikonen, 1997; Brown & London, 1998; Jacobson & Dietrich, 1999). These studies indicate that the fluid mosaic model by Singer and Nicholson (1972) has to be greatly modified to understand the structure and function of biological membranes.

A critical issue in the understanding of membrane domains is the realization that these domains are not static structures, particularly in the following two points. The domain may be formed and disintegrate continually, with lifetimes ranging from nanoseconds, seconds, and up to hours (Subczynski, Antholine, Hyde & Kusumi, 1990; Pasenkiewicz-Gierula, Subczynski & Kusumi, 1991; Gaidarov, Santini, Warren & Keen, 1998; Adams & Nelson, 1998). Second, even in the long-lived domains, the constituent molecules may be rapidly exchanging. Some molecules go out while other molecules come in, just like micelles, in which constituent molecules move in and out continuously (Simons & Ikonen, 1997; Kawasaki, Yin, Subczynski, Hyde & Kusumi, 2001). Because of this, the molecular organization and dynamics of such structures were investigated by us in the ps-to-μs regime, using spin-labeling techniques. We studied (1) the hydrocarbon chain order in the 100 ns time regime, (2) the effective reorientation time of the hydrocarbon chains in the time regime 100 ps and 10 ns, (3) the hydrophobicity profiles across the membrane, and (4) the local diffusion-solubility characteristics of oxygen in the membrane, which are sensitive to the molecular dynamics up to several μs.

Molecular oxygen has a unique characteristic as a membrane probe: its small size and appropriate level of hydrophobicity allow it to enter the small vacant pockets that are transiently formed in the lipid bilayer membranes. Therefore, the collision rates between oxygen and nitroxide spin labels placed at specific location in the membrane are sensitive to the dynamics of *gauche-trans* isomerisation of lipid hydrocarbon chains and to the structural nonconformability of neighboring lipids (Träuble, 1971; Pace & Chan, 1982; Subczynski, Hyde & Kusumi, 1991; Altenbach, Greenhalgh, Khorana & Hubbell, 1994). The DOT method (the method of discrimination by oxygen transport) developed by us (Ashikawa, Yin, Subczynski, Kouyama, Hyde & Kusumi, 1994) for analysis of saturation recovery EPR spin labeling data using molecular oxygen as a probe was successfully used to study reconstituted model membranes and biological membranes, with special attention paid to the membranes crowded with integral proteins or single transmembrane α-helical peptides.

Using the DOT method, we established that in reconstituted membranes of bacteriorhodopsin (BR) for a low BR/lipid ratio (1/80), the lipid environment is homogenous on a $0.3 - 10$ μs

scale. In the presence of molecular oxygen up to 50% of atmospheric air, only single exponential saturation-recovery EPR signals were observed (Ashikawa *et al.*, 1994). Because it is evident that the bulk and boundary regions around BR molecules coexist in this sample (East, Melville & Lee, 1985; Ryba, Horváth, Watts & Marsh, 1987; Horváth, Brophy & Marsh, 1988; Marsh, 1997), the exchange rate of lipids between the bulk and the boundary regions must be much greater than this time range. It also indicates that the oxygen collision rate cannot differentiate the bulk and protein-boundary regions. Therefore in the following, the bulk-plus-boundary regions will be indicated as the BULK domain for simplicity. Also, in 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) membranes containing a high amount of a transmembrane α-helical peptide (Ac-$K_2L_{24}K_2$-amide or Ac-$K_2(LA)_{12}K_2$-amide at 10 mol%), saturation recovery EPR detected the presence of a single homogenous membrane environment for both peptides. This suggests that peptides are well dispersed, and that POPC is exchanging rapidly between the boundary and bulk domains (Subczynski, Lewis, McElhaney, Hodges, Hyde & Kusumi, 1998).

At a high BR/lipid ratio (1/40), we established the presence of a specific lipid domain that has a slow oxygen transport rate (SLOT domain). Two exponential saturation-recovery EPR signals were observed in the presence of molecular oxygen (Ashikawa *et al.*, 1994). This was possible because the exchange rates of spin-labeled lipids between the SLOT domain and the BULK domain were much smaller than 10^5 s^{-1} (T_1^{-1} in the absence of oxygen).

In reconstituted model membranes containing BR or α-helical transmembrane peptides, the recovery rate of both one and two-component saturation-recovery signals increases proportionally to the partial pressure of oxygen. However, in the influenza virus (IFV) membrane where two-component saturation-recovery signals of a fatty acid spin label were observed, the recovery rate did not increase in proportion to the partial pressure of oxygen. This suggests the presence of another pathway that modifies electron spin relaxation. Therefore, we developed a theory that can deal with saturation recovery in the presence of two domains with different oxygen transport rates and lipid exchange between them (Kawasaki *et al.*, 2001). We used that theory to obtain characteristics of the SLOT domain and the lipid exchange rates between the SLOT domain and the BULK domain in the IFV membrane. We proposed that the SLOT domain in the viral mem-

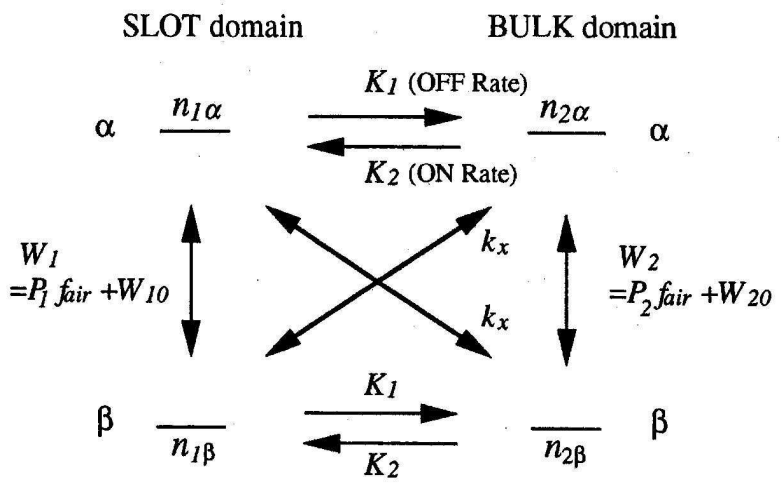

Fig. 1. A scheme for analyzing saturation-recovery signals of spin labels in the presence of two domains that possess different oxygen transport rates: the SLOT domain and the BULK (bulk plus boundary) domain. An important point in this scheme is that it includes exchanges of lipid-type spin labels between the two domains (K_1 and K_2, outbound and inbound rates of the SLOT domain, respectively). We assume that all spin label molecules are available for the exchange processes. In addition, this scheme includes the following two relaxation processes. 1) Electron spin transition of the spin label in each domain; the transition rates W_1 and W_2, are linear functions of the partial pressure of air, f_{air}, in the equilibrating gas mixture, W_{10} and W_{20} are the electron spin transition rates at $f_{air} = 0$, and $2P_1$ and $2P_2$ are the rates of oxygen collision with the spin label in a sample equilibrated with air. 2) Heisenberg exchange between the spins in different domains (k_X is the Heisenberg exchange rate). The three states of the doxyl nitrogen nuclear spin are assumed to mix into a single state because of fast nuclear spin relaxation (Popp and Hyde, 1982; see also Yin et al., 1987). The n values represent the instantaneous spin populations per unit volume of the four levels.

brane is the cholesterol-rich raft domain stabilized by the trimers of hemagglutinin and/or the tetramers of neuraminidase.

BIMOLECULAR COLLISIONS

If it is possible to put a spin label at specific labeled sites or in restricted domains such as a membrane, the bimolecular collision frequency with oxygen in this specific environment will be recorded. In the methodological approach described here, the bimolecular collision rate between oxygen (paramagnetic, a fast relaxing species) and the nitroxide spin label (a slow relaxing species) placed at specific locations in the membrane is evaluated in terms of an oxygen transport parameter ($2P$) using a saturation-recovery EPR technique (Kusumi, Subczynski & Hyde, 1982; Subczynski, Hyde & Kusumi, 1989; Subczynski *et al.*, 1991). $2P$(x) at x location in the membrane is defined as

$$2P(\mathrm{x}) = T_1^{-1}(\mathrm{x}, air) - T_1^{-1}(\mathrm{x}, N_2) \quad (1)$$

where the T_1s are the spin-lattice relaxation times of the nitroxide in samples equilibrated with atmospheric air and nitrogen, respectively. $2P$(x) is proportional to the rate of collision between the spin label and molecular oxygen. According to the Smoluchowski equation, the collision rate is thus proportional to the product of the local oxygen concentration [O_2(x)] and the local translational diffusion of oxygen D_O(x) in a membrane that is equilibrated with atmospheric air;

$$2P(\mathrm{x}) = AD_O(\mathrm{x})[O_2(\mathrm{x})], \quad A = 8\pi p r_o \quad (2)$$

where r_o is the interaction distance between oxygen and the nitroxide radical spin labels (4.5 Å; Windrem & Plachy, 1980) and p is the probability that an observable event occurs when a collision occurs, and is very close to 1 (Hyde & Subczynski, 1989).

The effect of oxygen on the spin-lattice relaxation of spin labels is generally much greater than the motional effects (Kusumi *et al.*, 1982). The oxygen transport parameter can be successfully used as a sensitive monitor of the membrane structure and dynamics (for review see Subczynski, 1999; Subczynski & Wisniewska, 2000). Profiles of the oxygen transport parameter across the membrane allowed us to understand movement of oxygen molecules within the lipid bilayer and to calculate the oxygen permeability coefficient across model and biological membranes (Subczynski *et al.*, 1989; 1991; Subczynski, Hopwood & Hyde, 1992; Ashikawa *et al.*, 1994). In this approach, x indicates different depths in the membrane at which EPR measurements were performed. It can be easily achieved by attaching the nitroxide moiety to different carbon atoms of the hydrocarbon chain in stearic acid or phosphatidylcholine molecules or to their polar head group.

SLOT DOMAINS

In membranes consisting of two lipid environments with different oxygen transport rates (BULK and SLOT domains) and the exchange rates of the spin-labeled lipids between these domains slower than 10^5 s^{-1}, the saturation-recovery signal will be a simple double-exponential curve with time constants of $T_1^{-1}(f_{\mathrm{air}}, \mathrm{SLOT})$ and $T_1^{-1}(f_{\mathrm{air}}, \mathrm{BULK})$.

$$T_1^{-1}(f_{\mathrm{air}}, \mathrm{SLOT}) = 2P_1 f_{\mathrm{air}} + T_1^{-1}(N_2, \mathrm{SLOT}) \quad (3)$$

$$T_1^{-1}(f_{\mathrm{air}}, \mathrm{BULK}) = 2P_2 f_{\mathrm{air}} + T_1^{-1}(N_2, \mathrm{BULK}) \quad (4)$$

Here "x" must be changed to the two-membrane domain, BULK and SLOT, and the depth fixed. The same lipid spin label is distributed between the BULK and SLOT domains. Because [O_2(x)] is proportional to the partial pressure of oxygen in the equilibrating gas mixture, we use the fraction of air in the gas mixture, f_{air}, used in actual experiments. $2P_1$ and $2P_2$ are oxygen transport parameters in each domain. Because these $2P$ values are the rates of collision between the spin label and molecular oxygen extrapolated to a sample equilibrated with 100% air, $2Pf_{\mathrm{air}}$ represents the collision rate in a sample equilibrated with a gas containing f_{air} air. In general, $T_1^{-1}(N_2, \mathrm{SLOT})$ and $T_1^{-1}(N_2, \mathrm{BULK})$ are close in the membrane and the presence of two types of lipid domains can often be clearly manifested only after introducing molecular oxygen in the sample (DOT method). The collision rate of molecular oxygen with the nitroxide group of the spin label can be quite different in the two domains.

This approach allowed us to detect the presence of specific lipid domains that exhibit a slow oxygen transport rate (SLOT domain). The SLOT domain was detected in BR reconstituted membranes at a BR/lipid ratio of 1/40, in which the oxygen transport rate was smaller by a factor of 5 than in the BULK region (Ashikawa *et al.*, 1994). This domain was thought to be protein rich, in which every lipid molecule is in contact with two proteins or with protein and boundary lipids (thus the lipids are sandwiched either between two proteins or between a protein and boundary lipids)

Table 1: Evaluation of rate constants ($\times 10^6$ s^{-1}) described in Fig. 1 observed at 30°C in IFV membrane (Kawasaki *et al*., 2001).

	IFV membrane	
	SLOT domain	**BULK domain**
Electron spin transition rate	$W_{10} = 0.08$	$W_{20} = 0.095$
Oxygen transport parameter	$2P_1 = 0.14$	$2P_2 = 2.2$
Lipid exchange rate	$K_1 = 0.077$	$K_2 = 0.046$

and its hydrocarbon chain motion is suppressed to the level of the gel-phase membrane. The exchange rate of lipids between this domain and the BULK domain was not observed. Recently two different oxygen transport rates were detected in DMPC membranes containing 20 mol% cholesterol (Subczynski, preliminary results). This fluid-fluid membrane microheterogeneity exists only up to 45°C and can be related to coexisting phases, the so-called liquid-ordered (l_o) and liquid-disordered (l_d) phases (Almeida, Vaz & Thompson, 1992). Two-component saturation-recovery signals of a fatty acid spin label were observed in the IFV membrane, indicating the presence of the SLOT domain (Kawasaki *et al.*, 2001). The saturation recovery signals exhibit, however, more complex behavior and this case will be analyzed in detail below.

EXCHANGE OF LIPIDS BETWEEN THE SLOT DOMAIN AND THE BULK DOMAIN

In the following section, all equations will be expressed using electron spin transition rates; i.e.,

$$W_{10} = \tfrac{1}{2} T_1^{-1}(N_2, \text{SLOT}) \tag{5}$$

$$W_{20} = \tfrac{1}{2} T_1^{-1}(N_2, \text{BULK}) \tag{6}$$

$$W_1 = \tfrac{1}{2} T_1^{-1}(f_{\text{air}}, \text{SLOT}) \tag{7}$$

$$W_2 = \tfrac{1}{2} T_1^{-1}(f_{\text{air}}, \text{BULK}) \tag{8}$$

A theory has been developed to include the exchange of lipid-type spin labels between two domains that possess different oxygen transport rates. The saturation-recovery signal of spin labels distributed between the two coexisting lipid domains in the membrane was analyzed based on the scheme shown in Fig. 1.

Following Yin, Pasenkiewicz-Gierula and Hyde (1987), a set of rate equations was set up (Kawasaki *et al.*, 2001) with the following solution:

$$\begin{aligned} I(t, f_{\text{air}}, N) = {} & I_1[1 - \exp\{-(A - B)t\}] + \\ & + I_2[1 - \exp\{-(A + B)t\}] \end{aligned} \tag{9}$$

$$A(f_{\text{air}}, N) = W_1 + W_2 + \tfrac{1}{2}(K_1 + K_2 + k_{\text{x}} N) \tag{10}$$

$$\begin{aligned} B(f_{air}, N) = \Big[& (W_1 - W_2)^2 + (W_1 - W)\{K_1 - K_2 + \\ & + (K_1 - K_2) k_x N / (K_1 + K_2)\} \\ & + \tfrac{1}{4}(K_1 + K_2 + k_x N)^2 \Big]^{1/2}. \end{aligned} \tag{11}$$

where $I(t, f_{\text{air}}, N)$ is the observed saturation-recovery signal, I_1 and I_2 are constants to be defined by initial conditions, and N represents the total number of spins per unit volume and is proportional to the number of spin probes incorporated in the membrane.

$$N = n_{1\alpha} + n_{1\beta} + n_{2\alpha} + n_{2\beta} \tag{12}$$

As discussed below, all the rate constants (W_{10}, P_1, W_{20}, P_2, K_1, K_2, and k_X) are determined by obtaining saturation-recovery signals at various partial pressures of oxygen (at low spin-label concentrations), and at various concentrations of the spin label without oxygen. Dependencies of A and B^2 on oxygen concentration can be determined by the following equations obtained from Eqs. 7, 8, 10, and 11 (assuming $N = 0$, i.e., for a low concentration of the spin label):

$$\begin{aligned} A(f_{\text{air}}) = {} & (P_1 + P_2) f_{\text{air}} + \\ & + W_{10} + W_{20} + \tfrac{1}{2}(K_1 + K_2) \end{aligned} \tag{13}$$

$$B(f_{air})^2 = (P_1 - P_2)^2 f_{air}^2 + (P_1 - P_2)[2(W_{10} - W_{20}) + (K_1 - K_2)] f_{air} + (W_{10} - W_{20})^2 + (W_{10} - W_{20})(K_1 - K_2) + \tfrac{1}{4}(K_1 + K_2)^2. \quad (14)$$

Dependencies of A and $(A - B)(A + B)$ on the spin label concentration, N, in the absence of oxygen ($f_{air} = 0$) can be obtained from

$$A(N) = \tfrac{1}{2} k_X N + W_{10} + W_{20} + \tfrac{1}{2}(K_1 + K_2) \quad (15)$$

$$(A(N) - B(N))(A(N) + B(N)) = W_{10} k_x N + \frac{W_{20}(W_{10} - W_{20})(K_1 - K_2)}{(K_1 + K_2)} k_x N + 4W_{10}W_{20} + 2W_{10}K_2 + 2W_{20}K_1. \quad (16)$$

All coefficients on the right side of Eqs 13-16 are determined by fitting the curves of A versus f_{air}, B^2 versus f_{air}, A versus N, and $(A - B)(A + B)$ versus N. Because there are only seven rate constants, all of them can be determined by solving these equations for coefficients.

The theory described above was applied to analyze two-component saturation-recovery signals of a fatty acid spin label observed in the IFV membrane (Kawasaki *et al.*, 2001). The rate constants that were evaluated are listed in Table 1. The oxygen transport rate in the SLOT domain is smaller than in the BULK domain by a factor of 16, which is a large factor. This suggests a possibility that the SLOT domain in the IFV membrane may not simply be a protein-rich region, but cholesterol rich and protein rich as well, because cholesterol can further reduce the oxygen collision rate (Subczynski *et al.*, 1989; 1991).

It is proposed that the SLOT domain in the IFV membrane is the cholesterol-rich raft domain stabilized by the trimers of hemagglutinin and/or the tetramers of neuraminidase. The exchange rates from and to the SLOT domain were estimated to be 7.7 and 4.6×10^4 s^{-1}, respectively (Table 1). This indicates that the residency time of lipids in the SLOT domain is substantially longer than in the boundary region, and suggests that the SLOT domain may play an important role in the function of the plasma membrane. Each SLOT domain may be small, but the entire SLOT domain occupies a substantial area in the IFV membrane. From the ratio of the inbound (K_2) to the outbound (K_1) rates of the lipid in the SLOT domain, the SLOT domains as a whole may occupy about one-third of the membrane $\{K_2 / (K_1 + K_2) = 0.046 / (0.046 + 0.077) = 0.37\}$.

FINAL REMARKS

It should be re-emphasized that the SLOT domain, which is a protein-rich raft domain in the IFV membrane, is a dynamic structure. Either the constituent lipid molecules stay in the SLOT domain and BULK domain for less than 20 μs (the inverse of K_2, the slower exchange rate) or these domains are constantly formed and dispersed at an average of every 20 μs. The DOT method developed for analysis of pulse EPR spin label data using molecular oxygen as a probe is useful in studies of the SLOT domains, particularly protein-stabilized cholesterol-rich raft domains, and the exchange of lipids between the SLOT domain and BULK domain.

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