ESR SPIN-LABEL STUDIES OF LIPID-PROTEIN INTERACTIONS IN MEMBRANES

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ABSTRACT Lipid spin labels have been used to study lipid-protein interactions in bovine and frog rod outer segment disc membranes, in (Na^+, K^+)-ATPase membranes from shark rectal gland, and in yeast cytochrome oxidase-dimyristoyl phosphatidylcholine complexes. These systems all display a two component ESR spectrum from 14-doxyl lipid spin-labels. One component corresponds to the normal fluid bilayer lipids. The second component has a greater degree of motional restriction and arises from lipids interacting with the protein. For the phosphatidylcholine spin label there are effectively 55 ± 5 lipids/200,000-dalton cytochrome oxidase, 58 ± 4 mol lipid/265,000 dalton (Na^+, K^+)-ATPase, and 24 ± 3 and 22 ± 2 mol lipid/37,000 dalton rhodopsin for the bovine and frog preparations, respectively. These values correlate roughly with the intramembrane protein perimeter and scale with the square root of the molecular weight of the protein. For cytochrome oxidase the motionally restricted component bears a fixed stoichiometry to the protein at high lipid:protein ratios, and is reduced at low lipid:protein ratios to an extent which can be quantitatively accounted for by random protein-protein contacts. Experiments with spin labels of different headgroups indicate a marked selectivity of cytochrome oxidase and the (Na^+, K^+)-ATPase for stearic acid and for cardiolipin, relative to phosphatidylcholine. The motionally restricted component from the cardiolipin spin label is 80% greater than from the phosphatidylcholine spin label for cytochrome oxidase (at lipid:protein = 90.1), and 160% greater for the (Na^+, K^+)-ATPase. The corresponding increases for the stearic acid label are 20% for cytochrome oxidase and 40% for (Na^+, K^+)-ATPase. The effective association constant for cardiolipin is ~ 4.5 times greater than for phosphatidylcholine, and that for stearic acid is 1.5 times greater, in both systems. Almost no specificity is found in the interaction of spin-labeled lipids (including cardiolipin) with rhodopsin in the rod outer segment disc membrane. The linewidths of the fluid spin-label component in bovine rod outer segment membranes are consistently higher than those in bilayers of the extracted membrane lipids and provide valuable information on the rate of exchange between the two lipid components, which is suggested to be in the range of 10^6–10^7 s^{-1}.

INTRODUCTION

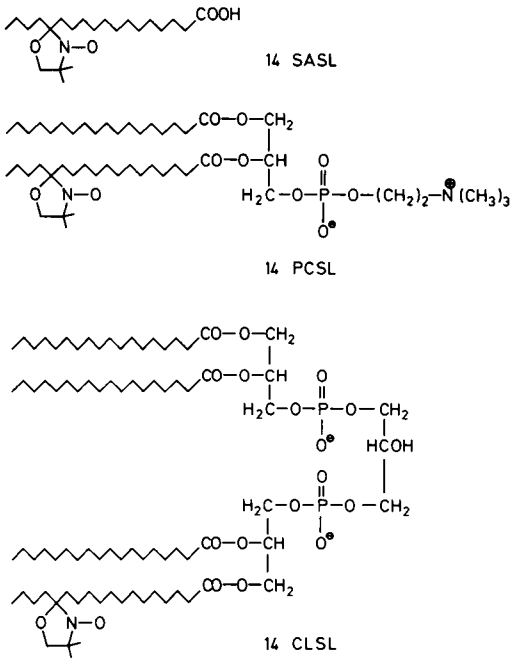
The electron spin resonance (ESR) spectra of spin-labeled lipid molecules provide a method of probing the molecular interactions at the protein-lipid interface in biological membranes (for a review see reference 1). Jost et al. (2) first reported the existence of a motionally restricted lipid spin-label component, in addition to the usual fluid lipid bilayer component, in membraneous cytochrome oxidase preparations. The proportion of this motionally restricted component depended directly on the protein content of the samples, and its amount correlated well with the approximate intramembrane perimeter of the protein. Therefore the motionally restricted component was attributed to the first shell or boundary layer of lipids surrounding the protein in the membrane. Subsequent work has focused on attempts to test the generality of these effects in different membranes, particularly those which have not been delipidated or reconstituted, and to delineate better the origin, structure and dynamics of the motionally restricted lipid component (1). These studies are still in progress.

In this paper we present data from new systems to compare with those already investigated by us. In particular we consider those boundary layer properties which are of special relevance to current discussions of lipid-protein interactions in membranes: the effects of protein aggregation on the stoichiometry of the motionally restricted component; the lipid selectivity of various proteins for the

motionally restricted component; and the rate of exchange of the motionally restricted lipids with the rest of the bilayer lipids. We address the following questions. (*a*) Does the motionally restricted lipid component arise from direct interaction of the spin-labeled lipid with the protein or merely from trapping of the lipid between proteins? (*b*) Do the properties of the motionally restricted lipids arise simply from occupancy of sites next to the protein, or is there some more specific molecular interaction? (*c*) Are the boundary layer lipids likely to be at exchange equilibrium with the bilayer lipids during a functional cycle of the protein?

MATERIALS AND METHODS

Cytochrome oxidase (CO) was purified from baker's yeast and the endogeneous lipids substituted by dimyristoyl phosphatidylcholine (DMPC) as described in (3). CO/DMPC complexes of defined lipid:protein ratio were prepared and characterized according to reference 3, and were suspended in 10 mM Tris, 1 M KCl, 1% (wt/vol) sucrose, pH 7.0 buffer. Specific activities were in the range 1,500–2,500 $min^{-1} \cdot mg^{-1}$ (referred to 1 ml assay volume) depending on lipid:protein ratio. Bovine and frog rod outer segment (ROS) disc membranes were prepared according to the method of Uhl et al. (4), and were suspended in 125 mM NaCl, 3.5 mM KCl, 14 mM glucose, 200 μM EDTA, 600 μM $CaCl_2$, 600 μM $MgCl_2$, 15 mM Hepes, pH 7.3 buffer. The A_{280}/A_{500} absorbance ratio was typically in the range of 2.0–2.2 for bovine and 2.3–2.4 for frog preparations. (Na^+, K^+)-ATPase (E. C. 3.6.3.1)-rich membranes were prepared from the rectal gland of *Squalus acanthias* as described in (5), and were suspended in either 20 mM histidine, 5% (vol/vol) glycerol, pH 7.2 buffer, or in 20 mM histidine, 100 mM NaCl, 1 mM CDTA, pH 7.2 buffer. Specific activities were typically 1,500 μmol $P \cdot mg^{-1} \cdot h^{-1}$. Membrane lipids were extracted with $CHCl_3$:CH_3OH 2:1 (vol/vol). For ROS lipids, argon saturated buffers and solvents were used; manipulations were performed under an argon or nitrogen atmosphere and 20 μg/ml butylated hydroxytoluene was added to all solvents to minimize lipid peroxidation. The stearic acid spin label, 14-SASL, was synthesized according to (6); the corresponding phosphatidylcholine, 14-PCSL, according to (7); and the cardiolipin spin-label, 14-CLSL, according to (8). Further details of the spin label syntheses are given in (1). Membranes were labeled at a level of ~ 1 mol/100 from a small volume of concentrated spin-label solution in ethanol, then washed to remove excess label and ethanol. ESR spectra were recorded on a Varian 9 GHz E-Line Century Series spectrometer (Varian Instrument Division, Palo Alto, CA), equipped with temperature regulation by nitrogen gas flow. Data collection and processing were performed with a PDP 11/10 dedicated computer (Digital Equipment Corporation, Maynard, MA) with LPS interface and VT-11 display. For further details of the spin-labeling and ESR techniques, see reference 9.



RESULTS AND DISCUSSION

Quantitation of Motionally Restricted Lipid

The ESR spectra of the 14SASL stearic acid spin-label in (Na^+, K^+)-ATPase membranes and in aqueous bilayers of the extracted membrane lipids are given in Fig. 1 *a*, *b*. A motionally restricted spin-label component is seen in the outer wings of the spectrum from the membranes (indicated by arrows in Fig. 1), which is not present in the spectrum from the lipids alone. Digital subtraction of the lipid spectrum from the membrane spectrum yields the spectrum of the motionally restricted component (Fig. 1 *d*), and double integration gives the fraction of the total spin-label intensity (34%) which is present in this component. The difference spectrum in Fig. 1 *d* indicates a considerably reduced mobility relative to that of the bilayer lipids at this temperature, and lies close to the limit of motional sensitivity of conventional ESR (see, e.g., reference 10). Subtraction of the motionally restricted component from the membrane spectrum, using a matching spectrum from lipid-depleted cytochrome oxidase at 40°C, yields the spectrum of the fluid bilayer component (Fig. 1 *c*) which is closely similar to that of the extracted lipids. Double integration yields a relative intensity of 66% for this component, in agreement with the quantitation from the motionally restricted component. Very similar results are obtained using the spectrum of dimyristoyl phosphaitdylcholine vesicles at 2°C, which has a slightly different lineshape, for the motionally restricted component.

Knowing the lipid:protein ratio, n_t, in the samples, it is possible to calculate the effective number of lipid molecules, n_1, associated with each protein molecule from the fraction of spin labels in the motionally restricted component. If the spin label reflects the unlabeled lipid distribution in an exact 1:1 fashion, and if further there is no selectivity between the different unlabeled lipids (3),

$$(n_f^*/n_b^*) = n_t/n_1 - 1 \qquad (1)$$

where (n_f^*/n_b^*) is the ratio of fluid to motionally restricted components in the spin-label spectrum. The values of n_1 for the (Na^+, K^+)-ATPase membrane and for frog rod outer segment disc membranes are given in Table I, in which they are compared with data we have previously obtained

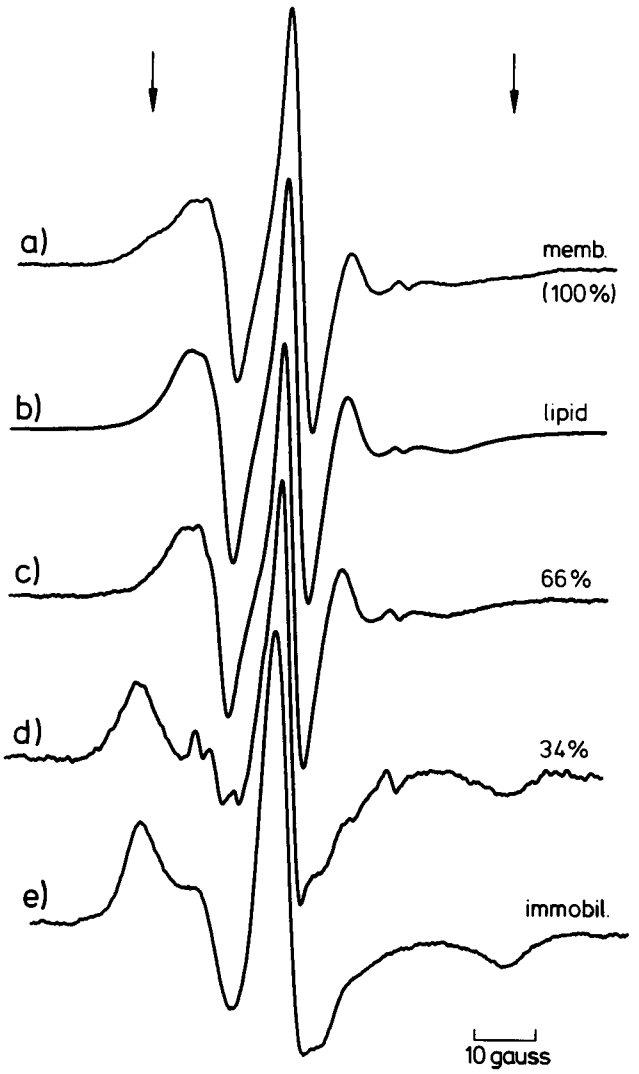


FIGURE 1 ESR spectra of the C14 stearic acid spin label, 14-SASL, in (Na^+, K^+)-ATPase membranes from *Squalus acanthias* rectal gland, T = 0°C. *a*, Membranes. *b*, Aqueous dispersion of extracted membrane lipids. *c*, Fluid component difference spectrum obtained by subtracting the immobilized spectrum given in *e* (33% relative intensity) from the membrane spectrum. *d*, Immobilized component difference spectrum obtained by subtracting the lipid spectrum (66% of the double-integrated intensity) from the membrane spectrum.

for yeast cytochrome oxidase-dimyristoyl phosphatidylcholine complexes (3, 11), bovine rod outer segment disc membranes (12, 13) and the acetylcholine receptor-rich membrane from *Torpedo marmorata* (14, 15). For cytochrome oxidase and the (Na^+, K^+)-ATPase membrane, some selectivity of the motionally restricted component is observed, depending on lipid type (see below). In these two cases, the values in Table I are for the phosphatidylcholine spin-label, which correspond to the values obtained with the majority of the spin-labels. In the majority of cases the values given in Table I remain essentially constant over a considerable temperature range

TABLE I

STOICHIOMETRIES OF THE MOTIONALLY-RESTRICTED LIPID SPIN LABEL COMPONENT IN VARIOUS LIPID-PROTEIN SYSTEMS

Protein/membrane	mol wt*	n_l^{exp}‡ mol/mol	$n_l^{exp}/\sqrt{\text{mol wt}}$ ($\times 10^3$)	n_l^{calc}§ (mol/mol)
Cytochrome oxidase -DMPC	200,000	55 ± 5	0.123 ± 0.011	50
Bovine rod outer segment disc/rhodopsin	37,000	24 ± 3	0.125 ± 0.016	24
Frog rod outer segment disc/rhodopsin	37,000	22 ± 2	0.114 ± 0.010	(24)
Na^+/K^+-ATPase shark rectal gland	265,000	58 ± 4	0.112 ± 0.008	(~60)
Acetylcholine receptor-rich membrane/*T. marmorata*	250,000	45%	—	52–55

*mol wt is the protein molecular weight.

‡n_l^{exp} is the effective number of motionally restricted lipids/protein deduced from the spin label experiments. The lipid/protein compositions are taken from the following references: cytochrome oxidase (3); bovine ROS (12); Na^+/K^+-ATPase (29); acetylcholine receptor (15). For frog ROS, lipid/protein ≈61 lipids/rhodopsin.

§n_l^{calc} is the estimated number of lipids which can be accommodated around the intramembraneous perimeter of the protein. Dimensional data and assumptions are referenced in the following: cytochrome oxidase (1–3); rhodopsin (1, 12); Na^+/K^+-ATPase (1); acetylcholine receptor (15). For the Na^+/K^+-ATPase the data are obtained from freeze-fracture electron microscopy and thus are regarded as approximate. The bovine rhodopsin value is assumed for frog rhodopsin.

and over a wide range of lipid:protein ratios (in the case of cytochrome oxidase-DMPC).

The value of n_l for cytochrome oxidase is in good agreement with the value of 47 lipids/cytochrome oxidase obtained originally by Jost et al. (2, 16) for bovine cytochrome oxidase. The value obtained for frog ROS disc membranes agrees very closely with that obtained previously for bovine ROS disc membranes. The species comparison is important in this case, since the motionally restricted component is best resolved at lower temperatures (cf., references 12, 13, and 17), and for the frog this coincides with the physiological temperature range. A previous controversy regarding the origin of immobilized lipid spin-label components in ROS membranes (12, 18–20) has recently been resolved (13), and it is now agreed that the results reported in Table I refer to rhodopsin in its normal state in the membrane. The result for the (Na^+, K^+)-ATPase membranes from *Squalus acanthias* rectal gland agrees quite closely with the value $n_l = 61 \pm 6$ which can be calculated from the data obtained in high salt by Brotherus et al. (21) for detergent-purified (Na^+, K^+)-ATPase from the electric organ of *Electrophorus electricus*, using rather different charged lipid spin labels. No experimental value of n_l is given for the acetylcholine receptor membranes because the preparations are not sufficiently enriched in the receptor protein to permit accurate comparison. It has been suggested (22) that the motionally restricted component in the receptor membranes arises from specific binding of fatty acid spin labels to the receptor. This has been shown not to be the case for

the results given here (15), since the motionally restricted component is observed with phosphatidylcholine, phosphatidylethanolamine and steroid spin labels alike.

The experimental values for n_1 in Table I are in quite good accord with the theoretical estimates of the first shell occupancies, based on the available data for the protein dimensions (see [1] for references). The relative values of n_1 are also found to equal the ratios of the square roots of the protein molecular weights, as might be expected for first shell occupancies if all three proteins protrude from the bilayer to the same extent. Thus, the original boundary layer interpretation can be extended from cytochrome oxidase to rhodopsin and the (Na^+, K^+)-ATPase. It should be emphasized, however, that with the present resolution of the dimensional data it cannot be decided whether the motionally restricted lipids occupy the entire surface of the protein, or are merely accommodated within surface invaginations.

Stoichiometry and Protein Aggregation

If the motionally restricted spin-label component represents the first shell of lipid surrounding the protein rather than lipid trapped between aggregating proteins, it should have a fixed stoichiometry with respect to the protein independent of the total lipid:protein ratio. For yeast cytochrome oxidase-DMPC complexes we have previously shown (3) that Eq. 1 is obeyed with $n_1 = 55 \pm 5$, for lipid protein ratios $n_t \geq 100$. For progressively delipidated bovine cytochrome oxidase, Jost et al. (2, 16) found that n_1 ~48, and Eq. 1 was obeyed down to $n_t \geq 48$. An important distinction is observed between the two systems. In the delipidated system the stoichiometry of the motionally restricted component is preserved down to the boundary layer ratio, whereas in the lipid-exchanged (cytochrome oxidase-DMPC) system the stoichiometry is reduced at the lower lipid:protein ratios, presumably as a result of protein-protein contacts induced by the randomizing effect of the cholate used to mediate lipid exchange (1, 3).

Analysis of the departures from stoichiometric ratios at low lipid:protein ratios can give information on the nature of protein aggregation and the affinity of the lipid for the protein. Let us assume that the protein-protein and protein-lipid contacts are purely random and similarly that there is no selectivity between lipids. Then a lattice theory for the protein and lipid occupancies gives the following expression for the fraction of fluid spin-label component (23):

$$(1-f) = \exp(-n_1/n_t) \qquad (2)$$

where f is the fraction of spin-label intensity in the motionally restricted component. Our data on cytochrome oxidase-DMPC complexes with the 14-PCSL phosphatidylcholine label are analysed according to this dependence in Fig. 2. The data at low lipid:protein ratios (with the exception of the lowest point) are reasonably consistent

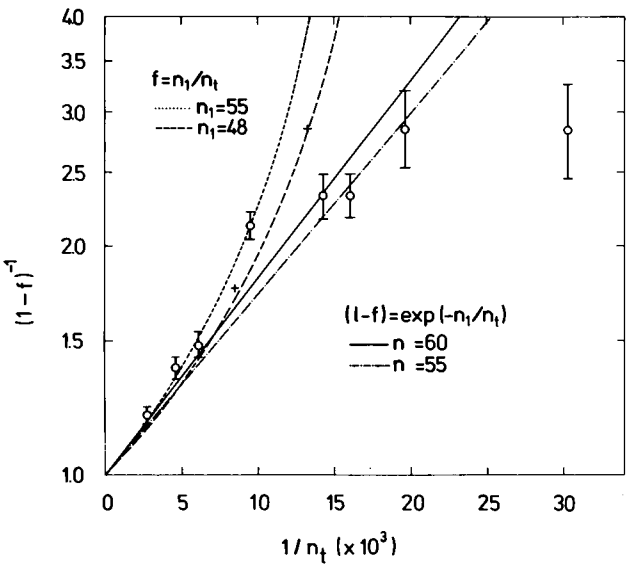


FIGURE 2 Lipid-protein titration of cytochrome oxidase complexes with allowance for protein-protein contacts. (- o -), data for yeast cytochrome oxidase-DMPC with 14PCSL label prepared by cholate-mediated exchange (3). (- + -), data for delipidated samples of membraneous bovine cytochrome oxidase (16, 25); (—), predicted dependence with random protein contacts for $n_1 = 60$; (-----), for $n_1 = 55$; (- -), predicted dependence with no protein contacts and $n_1 = 48$; (···), with $n_1 = 55$.

with a value of n_1 ~60 ± 5, assuming a reduction in the stoichiometry of the motionally restricted component arising from purely random contacts between the protein molecules. This gives further support for the value of $n_1 = 55 \pm 5$ obtained previously (3) from the data at high lipid:protein ratios. At high lipid:protein ratios the probability of random protein-protein contacts is low and so the two methods of analysis yield rather similar results: $n_1 \approx$ 55–60. At the lower lipid:protein ratios ($n_t < 100$) the random approximation is definitely more favored. The data are certainly inconsistent with the motionally restricted component arising from trapping of spin labels between aggregated protein; the opposite is true. It has previously been shown that the effect of high protein packing density is to cause a broadening of the fluid component rather than an increase in the immobilized component (24).

Lipid Selectivity

The ESR spectra of the cardiolipin, 14-CLSL; stearic acid, 14-SASL; and phosphatidylcholine, 14-PCSL, spin-labels in three different membrane systems are given in Fig. 3. These show a degree of specificity of the motionally restricted component for the various lipids, and also a different pattern of selectivity for the different proteins. The fractions, f, of motionally restricted lipid component in each spectrum have been obtained by spectral subtraction, and are given in Table II. If the specificity is modeled in terms of a lipid exchange with association constant, K_a,

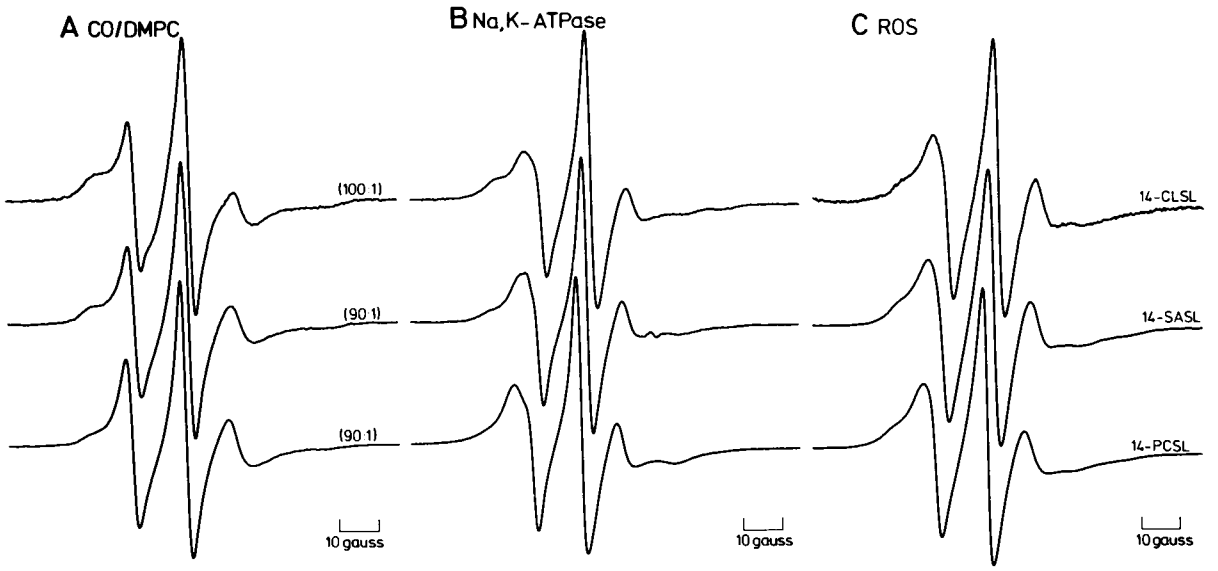


FIGURE 3 ESR spectra of the cardiolipin spin-label 14-CLSL (top row), stearic acid spin label 14-SASL (middle row) and phosphatidylcholine spin label 14-PCSL (bottom row) in: *A*, cytochrome oxidase-dimyristoyl phosphatidylcholine complexes of the indicated lipid:protein mol ratios, at $T = 32°C$; *B*, Na^+, K^+-ATPase membranes at $T = 8°C$; (*C*), bovine rod outer segment disc membranes at $T = 5°C$ (top row), $T = 10°C$ (middle row), $T = 7°C$ (bottom row).

for the spin label relative to the background lipid, then (11, 25):

$$(n_f^*/n_b^*) = n_t/(n_1 K_r) - 1/K_r. \quad (3)$$

The ratios of the association constants relative to PC and assuming that the number of sites n_1 is the same for each lipid, are given in Table II. The slight differences in lipid:protein ratio of the CO/DMPC complexes have been ignored in this calculation. The specificities for cardiolipin would be even higher if allowance were made for this (approximate corrected values are: $f = 0.82$, $K_r^L/K_r^{PC} = 4.8$). A general increase in the affinity of the sites is just one way of modeling the selectivity. Another likely possibility (although more difficult to quantitate) is the existence of a subclass of high affinity, possibly even totally specific, sites for particular phospholipids.

The values in Table II indicate a greater preference for stearic acid and for cardiolipin than for phosphatidylcholine in the first lipid shell of both cytochrome oxidase and the (Na^+, K^+)-ATPase. The most marked selectivity is for cardiolipin, but this is not an inevitable feature of the cardiolipin structure, as no selectivity is found with rhodopsin. This agrees with previous results (12) that demonstrated a lack of lipid selectivity in ROS membranes. A cardiolipin specificity has previously been reported for cytochrome oxidase in combination with different lipids (11, 26). However, the specificity is not unique to this protein, even though cardiolipin is peculiar to the inner mitochondrial membrane and frequently copurifies with cytochrome oxidase. The specificity of cardiolipin for the (Na^+, K^+)-ATPase is at least as great for cytochrome oxidase, even though this lipid is completely absent from the (Na^+, K^+)-ATPase membrane. The selectivity of the (Na^+, K^+)-ATPase for negatively-charged lipids is in agreement with previous observations with single-chain lipids (21). The present results extend these findings to phospholipids.

TABLE II
SELECTIVITY OF INTERACTION OF SPIN-LABELED CARDIOLIPIN, STEARIC ACID AND PHOSPHATIDYLCHOLINE WITH CYTOCHROME OXIDASE, Na^+, K^+-ATPASE AND RHODOPSIN

	CO/DMPC		Na^+, K^+-ATPase		ROS	
	f	K_r^L/K_r^{PC}	f	K_r^L/K_r^{PC}	f	K_r^L/K_r^{PC}
14-CLSL	0.74	3.0	0.50	4.3	0.28	0.8
14-SASL	0.59	1.5	0.28	1.7	0.34	1.1
14-PCSL	0.49	1.0	0.19	1.0	0.32	1.0

f, fraction of motionally restricted spin label; K_r, relative effective association constant, assuming a constant number of sites.

The lipid selectivities for the motionally restricted lipid spin-label component indicate a degree of molecular specificity in the lipid-protein interactions, beyond that of simple occupancy of the first shell lipid sites next to the protein. They also demonstrate that the motionally restricted component cannot arise solely from the trapping of spin label between proteins nor from a specific interaction between the nitroxide group of the spin label and the protein.

Exchange

Estimates of the mobilities of the motionally restricted spin-label components in cytochrome oxidase and acetyl-

choline receptor membranes have yielded correlation times $\tau \sim 50$ ns (3, 14). This mobility may represent segmental motion relative to the protein, or, alternatively, exchange between the first shell sites and the fluid bilayer. An upper estimate for the exchange rate would thus be $\nu_{ex} \sim 2.10^7$ s^{-1}. Similar results have recently been obtained for the motionally restricted component in ROS membranes (17).

Exchange between the two components at the above rate might be expected also to have an effect on the more fluid component. The low field linewidths of the fluid component in the spectra of the 14-SASL spin-label in bovine ROS membranes are compared with those from aqueous dispersions of the extracted membrane lipids in Fig. 4. Proton dipolar-decoupled, broad line ^{31}P NMR spectra of the total lipid dispersion showed this to be predominantly lamellar, with relatively little contamination from hexagonal phase lipid, throughout the whole temperature range. (The data given in Fig. 4 are measured directly from the spectra of the membranes. Closely similar results are obtained from the fluid component difference spectra, especially at the higher temperatures.) ESR results similar to those of Fig. 4 were obtained with an aqueous dispersion of the purified membrane phospholipids, prepared according to reference 27, and this was found by ^{31}P NMR to be completely lamellar throughout the temperature range studied. Thus we conclude that the results for the lipid dispersions in Fig. 4 should be directly comparable with those for the membranes.

The linewidths in Fig. 4 are measured by two methods.

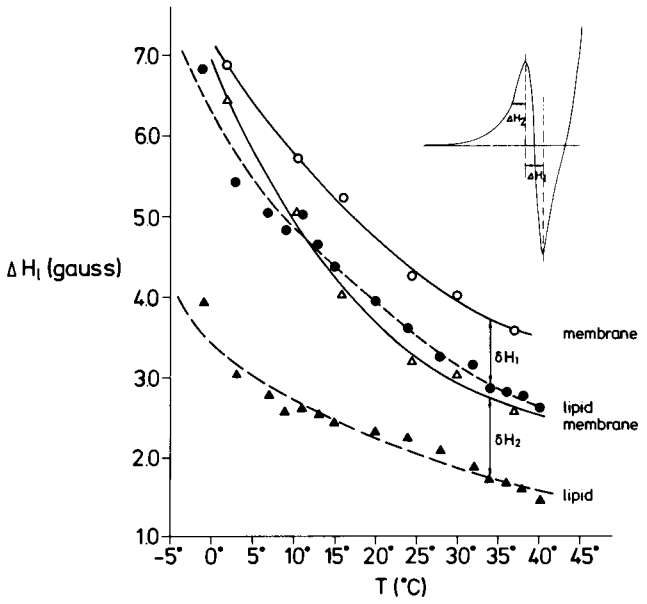


FIGURE 4 Low-field linewidths of the 14-SASL stearic acid spin-labels (see insert) in bovine rod outer segment membranes (— o —, — Δ —), and aqueous dispersions of the extracted membrane lipids (-- ● --, -- ▲ --), as a function of temperature. Circles represent the peak-to-trough linewidth, ΔH_1; triangles represent the half-width at half-height of the low-field peak, ΔH_2, as indicated in the insert.

The first is as a simple first-derivative line and should be most applicable at high temperatures. The second is with allowance for residual hyperfine anisotropy according to reference 6. Regardless of the method of measurement we found that the linewidths of the fluid component are consistently larger in the membranes than in the extracted lipid dispersions. This increased linewidth could be due either to a longer range (static) effect of the protein on the lipids beyond the first shell (3), or to exchange (see, e.g., reference 28), or due to both effects. An approximate value for the exchange rate can be obtained from the difference in linewidths. If it is assumed that this is due solely to exchange, then $\nu_{ex} \sim g\beta \cdot \delta H$ (28). The two different methods of measuring the linewidth in Fig. 4 give rather similar values, $\delta H_1 \simeq 0.8$ G and $\delta H_2 \simeq 1.0$ G at high temperatures at which the exchange is likely to have its most pronounced effect. This would require an estimated exchange rate of $\nu_{ex} \sim 2\text{–}3 \times 10^6$ s^{-1} (an upper limit, assuming the "static effect" to be nonfluidizing).

The estimates made from the two different spin-label components in the membrane are consistent in that the estimate from the motionally restricted component is an upper limit, and both lie within an order of magnitude of one another. A further feature of the spectra which is consistent with a relatively rapid exchange is that as the temperature increases it becomes progressively more difficult to perform subtractions such as those of Fig. 1 *d*. This may be because of the technical difficulties of matching the rather narrow linewidths of the fluid component at these temperatures, but a further possibility is that the spectra are entering a region of intermediate exchange rate and thus can no longer be approximated by two independent components. The critical exchange rate for this condition would be $\nu_{ex} \lesssim (A_{zz} - a_0)/h \approx 5 \times 10^7$ s^{-1}. Devaux and co-workers (30) have performed spectral simulations for two-site exchange between an immobilized and a fluid lipid component and have concluded that the spectra of covalently attached spin-label chains are consistent with exchange frequencies in the range 10^6–10^7 s^{-1}.

Thus it seems likely that the boundary lipids in ROS membranes may exchange at rates $\nu_{ex} \sim 10^6$–10^7 s^{-1}. These rates are not much slower than the rate of lipid exchange by lateral diffusion in fluid lipid bilayers (see, e.g., reference 10), and are faster than most enzymatic or transport cycles. The lipid selectivities in Table II imply a slower rate of exchange for cardiolipin or stearic acid, in association with cytochrome oxidase or the (Na^+, K^+)-ATPase, than for phosphatidylcholine. Even if the on-rate constant in these systems were diffusion-controlled, the off-rate for cardiolipin would be at least three to four times slower. Nonetheless, it is likely that the boundary layer will be at an exchange equilibrium during a functional cycle of the protein, and any effects of the lipid on activity will be determined by the fractional occupation of the first shell sites by the various lipids and by their conformation when they are in contact with the protein.

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DISCUSSION

Session Chairman: Thomas E. Thompson *Scribe*: Joy G. Mohanty

FEIGENSON: The lipids for which you measure binding constants have very different molecular areas (e.g., compare cardiolipin and stearic acid). The difference in areas could mean that different numbers of these lipids surround the proteins. Can you take into account the different molecular areas when you calculate binding constants?

MARSH: If you compare stearic acid and the phosphatidylcholine molecule, you find that one has roughly twice the molecular area of the other. If you think of the boundary layer, the stearic acid molecule has twice the opportunity to occupy sites on the surface; but the fluid lipid region also has twice the opportunity to occupy sites in that region. So I think that, to a first approximation, the effects of molecular area should cancel out.

However, there are differences not only in the molecular areas but also in the molecular structure. It is quite possible that the association of fatty acids with the protein can be different from those of phospholipids. As seen in Fig. 1, cardiolipin has a different structure, and so its association with the protein can be different. But the preferential association of cardiolipin is not purely a consequence of the different molecular structure because there is no selectivity. There may be a stronger binding in the case of stearic acid in the Na^+, K^+-ATPase membranes. This fact comes from the temperature dependence of the effective fatty acid association relative to that of the phospholipid molecule.

FEIGENSON: You observed binding-constant differences. That could imply specific sites on the protein. If there are specific sites, it is possible that cardiolipin might fill these up with fewer molecules, for example, in positively-charged regions. You might see these sites being filled up by cardiolipin. That would create a difficulty in interpreting your binding constants.

MARSH: Yes, if you really assume specific sites rather than primarily occupancy then I agree, that is possible. But then I do not think we have a similar situation. There could be a specific site for a stearic acid molecule but not for a phospholipid. However, we do not have enough experimental data at the moment to answer that.

FEIGENSON: For which of the lipids did you observe selective binding? The stearic acid and the cardiolipin could both be negatively charged. Is the stearic acid definitely negatively charged under the conditions of your experiment? Did you run your experiment with high salt concentration? Did you measure the actual binding constants at high salt concentration?

MARSH: The conditions are different in different preparations. We have not yet done a systematic salt-dependence study. We ran samples in the buffers that normally keep the proteins active. The samples of cytochrome oxidase were run in high salt, i.e., 1.0 M KCl, whereas Na^+, K^+-ATPase was run in low ionic strength. Thus, I think that the electrostatic interactions should be predominantly screened out in the case of cytochrome oxidase. We have data on the other phospholipids. A paper on cytochrome oxidase will appear shortly in *Biochemistry*. We see maximum preferential association for cardiolipin. Then come stearic acid and phosphatidic acid, which are comparable. For phosphatidylserine and phosphatidylglycerol, we see little selectivity and little more specificity than in the case of phosphatidylcholine and phosphatidylethanolamine. Therefore, it is not purely an electrostatic effect in the case of cytochrome oxidase.

DEBER: Table I, shows that 55 lipids surround cytochrome oxidase, 24 lipids surround rhodopsin and so on. I think it is very difficult to relate these numbers specifically to something that is expected just from conformational or topological or surface-area requirements of the proteins. For example, molecular weight differences are 200,000 vs. 37,000, i.e., a factor of ~6, but the number of lipids associated is 55 vs. 24. I think that the electrostatic factor which you mentioned must play some role in this. I would like to comment further that it may be useful to think of proteins in terms of some sort of macromolecular cations or anions for which there has to be an internal compensation of charge as a primary event when the protein and lipid are first mixed together. Thus, for example, if you look at the primary sequence of cytochrome oxidase, you will find it has a certain number of lysines or arginine residues that need to have counterions, which may originally be Cl^- or whatever but now are being replaced by something like phosphatidylcholine or cardiolipin or stearic acid, or phosphatidic acid anions. The reason for selectivity relates to these electrostatic forces: negatively-charged lipids would be more strongly associated with the protein than phosphatidylcholine or phosphatidylethanolamine since these themselves are neutralized molecules. I suggest some kind of quantitative correlation between the number of boundary or associated lipid molecules and the number of charged protein residues. Once these charge-compensated protein-lipid complexes are formed, other types of interactions may occur as well. Lipids themselves can form stabilized hydrophobic interactions with the hydrophobic residues of the protein. Would you comment on this?

MARSH: The values in Table I represent zwitterionic lipids, e.g., phosphatidylcholine or phosphatidylethanolamine. Negative charges do not come into play in these situations. That is why I said that where there is a specificity of binding, the values correspond to the lowest specificity. Further I would like to repeat that the experiments on cytochrome oxidase were conducted at high ionic strength, and hence we believe that the electrostatic interactions are suppressed.

PADDY: I would like to refer to your relative binding constants in Table II for different spin labels. I think the significant result is not the specificity itself but the magnitude of specificity when you are talking about a threefold difference in affinity. For instance, from your data in a membrane preparation containing one-quarter part cardiolipin and three-quarters phosphatidylcholine, both would have equal probability of occupying sites on cytochrome oxidase. We should keep in mind that many of these lipids which might preferentially associate with membrane proteins are at relatively low concentrations in the membrane. Because the relative affinities being measured are not a factor of 1,000 or 10,000, but something quite low, it may be that in the native membrane you will not see any preferential binding.

MARSH: There are two comments to make. One is that we do not have enough experimental data to address fully the point you are making. Second, this is an effective average association constant. In fact, there may be fewer, more specific, binding sites rather than a general, smaller increase in the association constant for all the binding sites.

POWELL: What do you think of the protein surface (40–55 lipid molecules/molecule cytochrome oxidase) where specific segregation of specific lipid (cardiolipin) occurs? Do a few specific sites (from one to three) with a high K_r get averaged over all 50 sites or does cardiolipin bind to all of them with the same affinity? (See Gwak and Powell, this volume.)

MARSH: We have not done enough experiments to answer. What we have to do (and it is not an easy experiment) is to vary the concentration of spin label relative to the total amount of lipid. One can only do this over a limited range because one is limited by sensitivity and by spin-spin interactions at the lower and upper bounds. Looking at your results (this volume), I believe that you see no association with phosphatidylcholine, but quite marked association of cardiolipin with cytochrome oxidase. If this is so, it points toward the possibility that there are specific sites rather than an average general increase in the association constant.

POWELL: We were surprised to find such high affinity of spin-labeled cardiolipin in detergent-solubilized preparations and such limited affinity for phosphatidylcholine. In our hands phosphatidic acid has almost but not quite as good affinity as cardiolipin. Of course that rules out the differences in structure between phosphatidic acid and cardiolipin which may influence association, with the exception that phosphatidic acid has two negative charges, as does cardiolipin. The best idea seems to be that there are some specific sites for cardiolipin mediated by the charge on the protein. We would like to think we are looking at the same cardiolipin molecules necessary for the activity of cytochrome oxidase as reported by Robinson et al. (1980. *Biochemistry.* 19:3656–3661).

MARSH: Certainly, these are very interesting results. We have also done experiments with different phospholipids, not just phosphatidylcholine, cardiolipin, and stearic acid. We do find a preference for phosphatidic acid, but it is not as high as cardiolipin. The preference is relatively low for phosphatidylglycerol or for phosphatidylserine. But I do not think we can directly relate these two experiments.

POWELL: We looked at the effect of detergent, high salt and pH on this effect. In the ranges employed, the charge on the cardiolipin does not change very much. Yeast cytochrome oxidase and bovine heart cytochrome oxidase could associate with these lipids in quantitatively different fashions.

YEAGLE: How useful is the square-root dependence of the molecular weight in the discussion of the population in the motionally-restricted environment when the proportion of the protein within the membrane is not the same from one protein to another?

MARSH: We showed in Table I that a regularity exists between the molecular weight and the number of motionally-restricted lipids. This is the regularity that you would expect if the proteins protruded from the membrane to roughly the same extent. This is certainly not a general observation. At least three other research groups have shown that the effective number of lipids associated with the Ca^{++}-ATPase is in the region of 20 to 25, whereas, on the basis of the square-root dependency, a value is predicted to be somewhere in the region of 35.

GUY: The spin label on the fatty acid has two oxygens on it. These could form H-bonds with the protein. Conceivably that can lead to two artifacts. Lipids may concentrate next to the protein. More importantly, it will immobilize the spin label on the tail. Do you have any evidence or rationale to say that these possible artifacts are negligible?

MARSH: My feeling is that the energetic requirements of protein folding will ensure that the protein folds in order to maximize its H-bonding capacity, and will not wait around for an ESR spectroscopist to introduce a spin label to fulfill its full complement of H-bonding. The experiment which addresses this problem is the protein:lipid titration where one varies the amount of unlabeled lipid relative to protein, as we did in the case of cytochrome oxidase. In this case we observed no preferential affinity of the spin labeled lipid compared to unlabeled lipid. In that situtation there is no strong specific interaction introduced by the spin label.

DRATZ: You clearly show in Fig. 4 the effect of bovine rhodopsin on the most mobile lipid. You show very nicely that at all temperatures, there is small but significant hindering of the motion of all the lipids. When you go to low temperature you pick up very distinctly a highly-hindered form. As you raise your temperature from the low end, presumably what happens is that the hopping rate of the lipids is picking up from next to the protein to the lipid further away. Is there evidence now that at a physiological temperature, for something like bovine rhodopsin, the lipid properties are not completely homogenized within your time window?

MARSH: Yes, if we stick with bovine rhodopsin at physiological temperature, the spectrum of the membrane is still not a simple single fluid motion of averaged components. It is either two-component or two-component-intermediate exchange. If we now go to frog rhodopsin, when the physiological temperature is lower one clearly gets two-component spectra which are in relatively slow exchange.

DRATZ: And what kind of exchange rates are you talking about?

MARSH: The exchange rates dominated by the anisotropy difference between those two-components and as in this text, $\sim 5 \times 10^7\ s^{-1}$.

DRATZ: That is very close to the lipid hopping rate in the absence of the protein, right?

MARSH: Yes; that is just the limit, not a measurement.

McINTYRE: In Fig. 1 of your paper you present spectral subtraction methods to deconvolute the complex spectrum of the spin label in the Na^+, K^+-ATPase membranes into two components: a fluid component and a motionally-constrained component. It is indeed comforting that both methods of subtraction, using the fluid component or the selected immobilized component, gave you the same answer. However, by simple analogy, if we were to prove that $2 + 5 = 7$, we would also expect that $5 + 2 = 7$. Such conclusions would not limit this to the only solution; $4 + 3 = 7$ and $1 + 6 = 7$ or other combinations are also possible. Do you believe that your spectral subtraction method gives an unique solution to the deconvolution?

MARSH: The mathematics required to describe spectral subtractions are rather more complicated than you suggest; one needs some sort of multidimensional representation. One starts with one spectrum and tries to decompose this into two. This is an undetermined problem. However, anyone who has done lots of these subtractions knows that if you try to match with a spectrum that is slightly off, then you quickly begin to get nonsense. So I think that when one has to fit the whole spectral shape, one comes very closely to a unique fit to these two components as seen in Fig. 1. In the case of cytochrome oxidase the situation is much more complicated; if you take a lipid spectrum at the same temperature, it does not at all correspond to the fluid component. There is also a distortion of the fluid component as a function of protein:lipid ratio, which corresponds to a decrease of the perturbation of the lipid away from the protein.

McINTYRE: You say that under certain circumstances you believe you can get a unique solution in that you can fit the whole spectrum. Under a variety of situations, I have been able to show that although we can fit the whole spectrum with two specific references, we can also select two different references and get an equally good fit to the whole spectrum. I agree that with many selected references, if you look at the derivative spectra, you can have a problem with mismatching. But obtaining a good fit to the whole spectrum does not prove that you have a unique solution to the deconvolution.

MARSH: I suppose this refers specifically to the spectra in your paper (McIntyre et al., this volume.) What you have done is to take the lipid at the same temperature and subtract it to what might be a good reasonable end-point from the membrane at that temperature. I feel that your end-point is not a single spectral component and that what you are doing is in fact isolating all the lipid populations which are to any extent perturbed by the protein. You are looking at not only the first shell but also at the subsequent shells. When you go to the lipid at a different temperature that more easily approximates the envelope of the outer shell distortions, you get something that approximates the first shell. If one does have subsequent shell distortions, then the problem has an undetermined answer.

McINTYRE: You are saying that in certain circumstances, particularly the spectra that I have been analyzing, a three-or-more-component analysis is equally valid.

MARSH: Yes, certainly for cytochrome oxidase with a multicomponent analysis, there are significant distortions beyond the first shell.

McINTYRE: In response to Ed Dratz's question and your answer that at least in one system at physiological temperature the two components are not readily resolvable, I have a further point.

In Fig. 3, you show a comparison of the spectra obtained for three different labels with three different membranes. It appears as though the motional parameters of the constrained species in the Na^+, K^+-ATPase and rod outer segments are similar to that of cytochrome oxidase spectra. However, these spectra are taken at very different temperatures (32°C vs. 5–10°C). Does this mean that the degree of motionally-constrained species is highly dependent on the membrane, especially when you note that the immobilized signal in Fig. 1 for Na^+, K^+-ATPase at 0°C matches the immobilized signal for cytochrome oxidase at 40°C?

MARSH: If I go to the first part of your question, I think you can make this comparison meaningfully only when you look at the difference spectra. And you must take it from me that if you compare the difference spectra for those three spectra of the top line in Fig. 3, they are in fact, significantly different. The line widths of the immobilized component in the Na^+, K^+-ATPase are somewhat narrower than those of cytochrome oxidase. I think you are probably right that there are significant differences which have not yet been explored in detail. It is difficult to give very firm answers to this problem for two reasons, one that it depends strongly on the technical quality of the difference spectra, second that these spectra are in the slow motional regime of the ESR spectroscopy. Quite subtle changes in the spectra can correspond to quite large significant changes in the mobility.

McINTYRE: How, in fact, do the difference spectra for the constrained species compare for the three systems at 30°C?

MARSH: I cannot answer that without looking at the spectra, but it is absolutely clear that those spectra are not in the 10^{-9} s regime.

BOGGS: We showed a few years ago, using differential scanning calorimetry with the intrinsic myelin protein, lipophilin, that it shows selectivity for acidic lipids. This is now being confirmed by Pat Jost and John Silvius using spin-labeled acidic lipids.

GRIFFITH: I just want to make a comment about the binding constants. We have to recalibrate our intuition about the equilibrium binding constants in membranes since we are used to thinking about equilibrium binding constants of substrates in aqueous solution. We have to remember that lipids in membranes are much more concentrated. For example, to achieve ~90% occupancy of a specific lipid binding site on the protein in a bilayer containing 10 mol % of a solute lipid requires a binding constant of ~100. For comparison, ~90% occupancy of a ligand-binding site on a water soluble protein in a dilute solution of aqueous ligand concentration $\sim 10^{-5}$M (a typical value) requires a binding constant of the order of 5×10^7. So a binding constant of the order of 100 in the membrane can be as significant as a binding constant of 5×10^7 in aqueous solution. (See J. R. Brotherus, et al. 1981. *Biochemistry*. 20:5261–5267.)

MARSH: This is very true, but we have not yet found an effective binding constant anywhere near 100.

GRIFFITH: An average relative binding constant (K_{av}) of 5 or 10 can correspond to a specific binding constant of the order of 100 when there are a large number of nonspecific binding sites contributing to the observed K_{av}.

MARSH: But that does not give a 90% occupancy of all of the sites, only of the specific sites. However, I agree with your viewpoint that the binding constants, although low, can be very significant in the context of membranes.