

Spin-Label Studies of Head-Group Specificity in the Interaction of Phospholipids with Yeast Cytochrome Oxidase[†]

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ABSTRACT: Yeast cytochrome oxidase complexes have been prepared in which $\geq 99\%$ of the endogenous lipid has been replaced by dimyristoylphosphatidylcholine, and the interaction with spin-labeled phospholipids of different polar head groups has been studied with electron spin resonance (ESR) spectroscopy. The ESR spectra of the different C-14-labeled phospholipids all consist of both a motionally restricted and a fluid lipid component, but the proportion of the two components varies with the different spin-label head groups for complexes of the same lipid/protein ratio. The more immobilized component represents lipid interacting with the protein, since it is not present in bilayers of the pure lipid. Similar proportions of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol have restricted chain motion, but a distinct preference is established for

cardiolipin (diphosphatidylglycerol) and phosphatidic acid, the greatest being for cardiolipin. For a complex containing 95 mol of dimyristoylphosphatidylcholine per mol of cytochrome oxidase, 80% more cardiolipin and 35% more phosphatidic acid than phosphatidylcholine are immobilized at 30 °C. As compared with previous lipid/protein titrations with the phosphatidylcholine spin-label [Knowles, P. F., Watts, A., & Marsh, D. (1979) *Biochemistry* 18, 4480-4487], the selectivity for cardiolipin and phosphatidic acid can be expressed as an increased relative binding constant: $K_r^{CL} \sim 5.5$ and $K_r^{PA} \sim 2$, respectively. Alternatively, the selectivity could be expressed as ~ 35 extra sites being available on the protein for cardiolipin molecules and ~ 15 extra sites per 200 000-dalton protein for phosphatidic acid molecules.

The interactions between integral proteins in membranes and the constituent membrane lipids are potentially important determinants of biological structure and function. Electron spin resonance (ESR)¹ studies of spin-labeled lipids in various membranes and lipid-protein systems have indicated the coexistence of two lipid spin-label populations, whose exchange rate is slow on the ESR timescale ($\nu_{ex} \leq 10^8$ s⁻¹). The first component corresponds to the normal, fluid lipid bilayer component in the membrane. The second component, which is not found in bilayers of the lipid alone, corresponds to lipids which are relatively immobilized on the ESR timescale ($\tau_c \geq 10^8$ s), and presumably arises from the lipid population whose chains are directly interacting with the hydrophobic surface of the integral membrane protein [see Marsh & Watts (1981) for a review and discussion of these results].

Previously, we have shown that this less mobile lipid spin-label component bears a constant stoichiometry to the protein in cytochrome oxidase-dimyristoylphosphatidylcholine complexes investigated with a phosphatidylcholine spin-label. The equivalent of 55 ± 5 lipids per 200 000-dalton protein were motionally restricted independent of the total lipid/protein ratio, the remainder being in the fluid component (Knowles et al., 1979). In addition, it was found from the lipid/protein titration that there was no selectivity between the labeled and the unlabeled phosphatidylcholine. The spin-label reflected the distribution of the host DMPC between the fluid and immobilized components in an exact 1:1 fashion. For these reasons, the less mobile lipid component was operationally defined as a first shell of lipid molecules interacting directly with the protein.

A question of considerable interest is whether there is any selectivity between the different phospholipid head groups in the composition of the first lipid shell, or whether the immo-

bilization of the lipid chains by the protein is relatively non-specific. In the present study, we have investigated the head-group specificity in the interaction of the motionally restricted lipid, again using the cytochrome oxidase-DMPC system and the same (C-14) positional isomer of the phospholipid spin-label, but with a variety of different phospholipid head groups. For complexes with the same lipid/protein ratio, the phosphatidylcholine spin-label then provides a fixed calibration relative to the host dimyristoylphosphatidylcholine.

Experimental Procedures

Materials. DMPC was obtained from Fluka (Buchs, Switzerland) and ran as a single spot on thin-layer chromatograms. Cholic acid, obtained from Sigma Chemical Co. (St. Louis, MO), was recrystallized twice from ethanol-water (2:1 v/v) before conversion to the sodium salt. The stearic acid spin-label, 14-SASL, was synthesized from the corresponding ketomethyl stearate essentially by the method of Hubbell & McConnell (1971). The phosphatidylcholine spin-label, 14-PCSL, was made by using the method of Boss et al. (1975). Other phospholipid spin-labels, 14-PESL, 14-PASL, 14-PGSL, and 14-PSSL, were made by enzymatic head-group exchange of the choline head group of 14-PCSL, catalyzed by phospholipase D (Ito & Ohnishi, 1974; Comfurius & Zwaal, 1977; Watts et al., 1979). The cardiolipin spin-label, 14-CLSL, was made essentially according to Cable et al. (1978), by reacylation of purified lysocardiolipin with 14-SASL using *N,N*-carbonylimidazole (Boss et al., 1975). After 2-3 days

[†] Abbreviations used: DMPC, L- β -dimyristoyl- α -phosphatidylcholine; 14-PASL, 14-PESL, 14-PCSL, 14-PSSL, and 14-PGSL, β -14-(4,4-dimethoxyazolidinyl-N-oxyl)stearoyl- γ -acyl- α -phosphatidic acid, -phosphatidylethanolamine, -phosphatidylcholine, -phosphatidylserine, and -phosphatidylglycerol, respectively; 14-CLSL, 14-(4,4-dimethoxyazolidinyl-N-oxyl)stearoylcardiolipin; 14-SASL, 14-(4,4-dimethoxyazolidinyl-N-oxyl)stearate; PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; CL, cardiolipin; ESR, electron spin resonance; TKS buffer, 10 mM Tris, 1.0 M KCl, and 1% (w/v) sucrose, pH 7.0, buffer.

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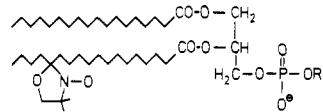
at 60 °C under vacuum, the major reaction product was purified on a basic silica gel column. All spin-labels were characterized by thin-layer chromatography and ESR. On thin-layer chromatograms in basic solvent systems, the spin-labels all cochromatographed with the parent unlabeled compounds. Detection was by molybdate spray followed by charring, and also by ninhydrin and Dragendorff's reagent where applicable. Spin-label was detected by quenching of the fluorescent indicator incorporated in the thin-layer plates. For further details of spin-label synthesis, see Marsh & Watts (1981). Spin-labels were kept in stock solutions of 4 mg/mL in ethanol, except for 14-PSSL which was in chloroform at 1 mg/mL.

Cytochrome oxidase was prepared from baker's yeast as described by Virji & Knowles (1978). The molecular weight of cytochrome oxidase was taken to be 200 000. The buffer used throughout was 1.0 M KCl, 10 mM Tris, and 1% sucrose (w/v) (TKS buffer) adjusted to pH 7.0 at 20 °C.

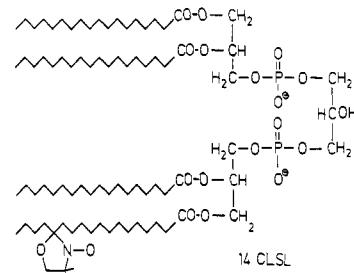
Methods. Substitution of the endogenous lipid of cytochrome oxidase by DMPC was carried out by using cholate-mediated exchange, as previously described (Knowles et al., 1979). A typical exchange medium contained 35 mg of cytochrome oxidase, 140 mg of DMPC, 700 µL of 2% cholate, and TKS buffer to make up a 70-mL final volume. After incubation at 25 °C for 45 min, 35 mL of Tris-KCl buffer was added, the mixture was cooled to 0 °C, and then 56.52 mL of saturated ammonium sulfate (pH 7.0, adjusted on a 1:6 diluted sample) was added dropwise with stirring at 0 °C. The mixture was left for 15 min at 0 °C and then centrifuged at 80000g for 10 min. The pellet was resuspended in a fresh portion (70 mL) of the exchange medium (140 mg of DMPC, 700 µL of cholate, and 69.3 mL of TKS buffer) and the procedure for the first exchange repeated. For preparation of samples with different lipid/protein ratios, the pellet after centrifugation of the ammonium sulfate precipitate from the third exchange was resuspended in 18 mL of the supernatant and divided into nine 2-mL portions for re-centrifugation. Each pellet was estimated to contain 1.7 mg of exchanged protein and was resuspended with minimum delay (\leq 15 min) in 0.5 mL of TKS buffer containing cholate to a final concentration of 0.5% and 0.6–1.6 mg of DMPC, or greater, depending on the final lipid/protein ratio required. The suspension was then incubated for 10 min at 25 °C and centrifuged (20 000 rpm, 10 min, Spinco 65 rotor), yielding a clear supernatant (volume \leq 2 mL) in all cases which was dialyzed against three 2-L changes of TKS buffer containing XAD 2 resin, at 15 °C for 40 h. [14 C]Cholate assays indicated that little residual cholate (7–15 mol per mol of protein, depending on the lipid/protein ratio) remained after dialysis.

Characterization of the DMPC complexes after dialysis was carried out as previously described (Knowles et al., 1979). All of the samples tested ran as a single band in sucrose density gradient centrifugation (4–60% sucrose in 0.1 M KCl–10 mM Tris buffer, pH 7.0). In addition, samples before dialysis in 1.5% cholate chromatographed as a single band on Sepharose 4B, indicating the absence of aggregated protein. The specific activity of the enzyme before dialysis was 2400 min $^{-1}$ mg $^{-1}$ and after dialysis was 1000–3000 min $^{-1}$ mg $^{-1}$, depending on the lipid/protein ratio. All activities were determined with 30 µM ferrocyanochrome *c* and are normalized relative to a 1-mL assay volume. The enzyme was incubated at 0 °C in TKS–0.5% cholate for 1 h prior to assay in all cases.

Samples were spin-labeled exogenously after dialysis, from a small volume of concentrated spin-label solution in ethanol. To obtain samples with identical lipid/protein ratios, a larger



R = H	14 PASL
R = $(\text{CH}_2)_2-\overset{\cdot}{\text{NH}_3}$	14 PESL
R = $(\text{CH}_2)_2-\overset{\cdot}{\text{N}}(\text{CH}_3)_3$	14 PCSL
R = $\text{CH}_2-\overset{\cdot}{\text{CH}}-\overset{\cdot}{\text{NH}_3}$ COO ⁻	14 PSSL
R = $\text{CH}_2-\overset{\cdot}{\text{CHOH}}-\text{CH}_2\text{OH}$	14 PGSL



14 CLSL

FIGURE 1: Phospholipid spin-labels of different head groups, labeled on the C-14 atom of the β chain [β -14-(4,4-dimethyloxazolidinyl- N -oxy)stearoyl- γ -acyl- α -phospholipid]. 14-CLSL, cardiolipin; 14-PASL, phosphatidic acid; 14-PGSL, phosphatidylglycerol; 14-PSSL, phosphatidylserine; 14-PESL, phosphatidylethanolamine; 14-PCSL, phosphatidylcholine.

preparation was divided into aliquots and spin-labeled separately with the various phospholipid labels.

Typically for the 95:1 samples, 10 µL of an ethanolic stock solution of the spin-label was added to a suspension of the complex (containing ca. 2 mg of DMPC and 6 mg of protein) in 10–13 mL of TKS buffer at 4 °C. After 5 min of incubation at 25 °C, the complex was centrifuged (10 min, 36000g) and transferred to a sealed-off 100-µL capillary. The complex was further concentrated in the capillary by centrifugation (5 min, 3000g) before ESR measurement. Results obtained by this labeling method for the 14-PCSL label were very similar to those obtained previously (Knowles et al., 1979) on incorporating the spin-label with the substituting lipid in cholate, prior to dialysis. For the 14-PSSL label, the same amount of complex as above was shaken briefly with a dried film of spin-label (0.02 mg) at room temperature. The sample was then diluted with 10–13 mL of the stock buffer and centrifuged (10 min, 36000g) to remove any spin-label vesicles. This washing procedure was repeated 2 further times and the pellet then prepared for ESR measurements as described above for the other labels.

ESR spectra were recorded on a Varian E-12 9-GHz spectrometer equipped with nitrogen gas flow temperature regulation. Samples were contained in the 1-mm-diameter sealed-glass capillaries within standard 4-mm-diameter quartz tubes containing light silicon oil for thermal stability. Spectra were digitized by using a Digital Equipment Corp. LPS system with a PDP 11/10 dedicated computer and analyzed with a VT-11 display by using interactive software written by Dr. W. Möller of this institute.

Results

The ESR spectra of the C-14 atom positional isomer of spin-labels with various phospholipid head groups (cf. Figure 1), all in cytochrome oxidase–dimyristoylphosphatidylcholine complexes of the same lipid/protein ratio, are given in Figure 2. The spectra are two-component in all cases. One component with narrow hyperfine splitting corresponds to a fluid

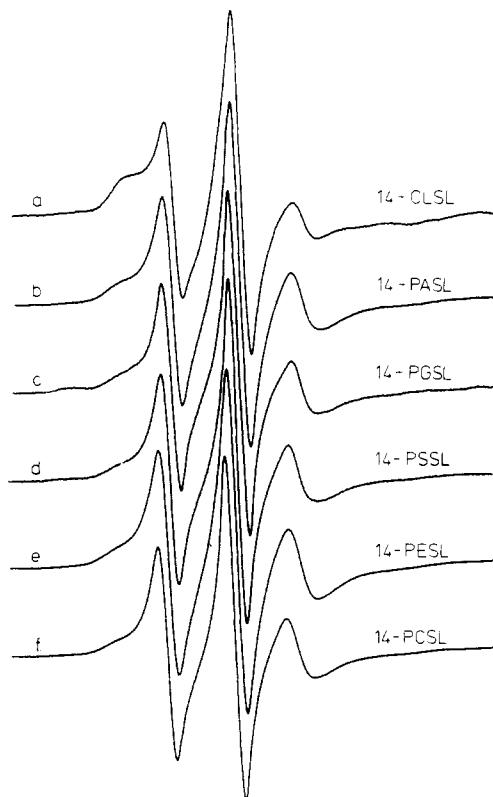


FIGURE 2: ESR spectra of different head-group spin-labels (see Figure 1) introduced exogeneously into separate aliquots taken from the same cytochrome oxidase-dimyristoylphosphatidylcholine complex of lipid/protein ratio 95:1, at $T = 32^\circ\text{C}$. Total scan width = 100 G.

bilayer lipid component. The other component with broader hyperfine splitting corresponds to lipids interacting directly with the protein, whose chain mobility is considerably restricted relative to that of normal bilayer lipids. This component is approaching the immobilized limit of spin-label motional sensitivity [cf. Knowles et al. (1979)].

Clear differences are observed between the spectra of certain labels. It can be seen that the cardiolipin (diphosphatidylglycerol) spin-label spectrum displays a considerably larger immobilized component than does the phosphatidylcholine spin-label spectrum in this complex. The phosphatidic acid spin-label spectrum also displays a somewhat larger immobilized component, whereas the spectra of the phosphatidylserine, phosphatidylglycerol, and phosphatidylethanolamine labels are all very similar to that of the phosphatidylcholine label. The ESR spectra of the various phospholipid labels in bilayers of dimyristoylphosphatidylcholine alone at 32°C are given in Figure 3. All the spectra consist of a single fluid component and are very similar for all the different labels. These control spectra demonstrate that the motionally restricted component in the spectra from the complexes arises from interaction with the protein and that the differences between the different labels arise also from interaction with the protein and not from any intrinsic differences in lipid fluidity or from lipid phase separations.

Clearly from the spectra of Figure 2, cytochrome oxidase shows a relatively strong selectivity for cardiolipin, a weaker selectivity for phosphatidic acid, and practically no selectivity for phosphatidylserine, phosphatidylglycerol, phosphatidylethanolamine, or phosphatidylcholine. The selectivity is seen very clearly when intersubtractions are performed between the spectra of the different labels [see Jost & Griffith (1978a) for a detailed discussion of spectral subtraction]. Subtracting the phosphatidylcholine spectrum from the cardiolipin spectrum

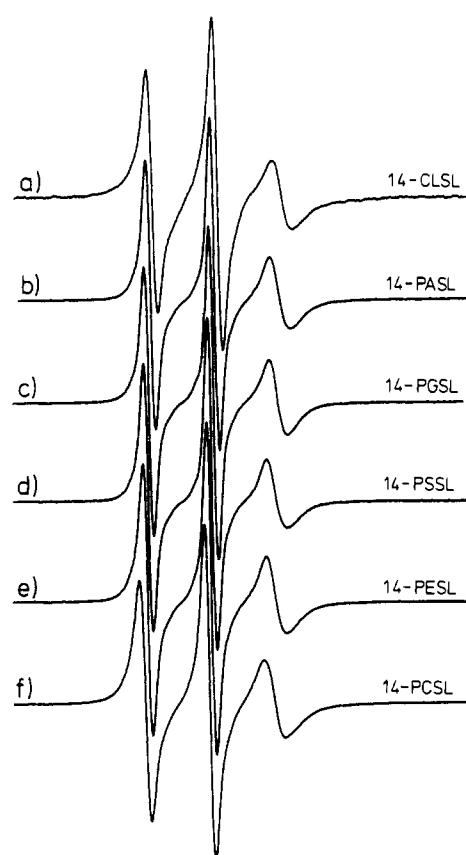


FIGURE 3: ESR spectra of different head-group spin-labels (see Figure 1) in bilayers of dimyristoylphosphatidylcholine in TKS buffer, $T = 32^\circ\text{C}$. Total scan width = 100 G.

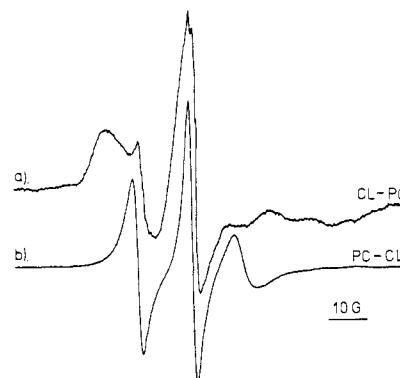


FIGURE 4: ESR difference spectra obtained by intersubtraction between the spectra of the 14-CLSL and 14-PCSL labels in cytochrome oxidase-dimyristoylphosphatidylcholine complexes of lipid/protein ratio 95:1, $T = 32^\circ\text{C}$. (a) 14-CLSL minus 14-PCSL, immobilized component end point; (b) 14-PCSL minus 14-CLSL, fluid component end point. Total scan width = 100 G.

gives a difference spectrum corresponding solely to a (complex) immobilized component, whereas subtracting the cardiolipin spectrum from the phosphatidylcholine spectrum gives a difference spectrum corresponding solely to a fluid component (see Figure 4). Qualitatively similar end points are obtained in intersubtractions between phosphatidic acid and phosphatidylcholine. Intersubtractions between phosphatidylserine, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine reveal essentially very little difference between the spectra of all these labels and yield a flat base line.

The quantitative end points of the intersubtractions can be used to determine the relative amounts of fluid and immobilized components in the two spectra being subtracted. This subtraction method has the advantage that the spectra refer

to exactly the same lipid-protein complex. Thus, the line shapes of the two components are more likely to match between the two spectra than if the spectrum of the pure lipid or another complex was used. Let α^A and α^B be the fractions of the immobilized component in the spectra from label A and label B, respectively, and $1 - \alpha^A$ and $1 - \alpha^B$ are the corresponding fractions of the fluid component. If f^A is the fraction of the spectrum from label B which must be subtracted from the spectrum from label A to give an *immobilized* end point and f^B is the fraction of the spectrum from label A which must be subtracted from the spectrum of label B to give a *fluid* end point, then² $f^A = (1 - \alpha^A)/(1 - \alpha^B)$ and $f^B = \alpha^B/\alpha^A$. Hence, the fractions of the immobilized component in the two spectra from labels A and B are $\alpha^A = (1 - f^A)/(1 - f^A f^B)$ and $\alpha^B = \alpha^A f^B$. These two expressions can thus be used to determine the fractions of both fluid and immobilized components in the two spectra from the intersubtraction end points f^A and f^B . [This method is essentially similar to that of Brotherus et al. (1980).]

The fractions of immobilized lipid in the cardiolipin and phosphatidylcholine spectra obtained from the intersubtractions in Figure 4 are $\alpha^{CL} = 0.82$ and $\alpha^{PC} = 0.46$, respectively. Similarly, intersubtractions between the phosphatidic acid and phosphatidylcholine spectra yield $\alpha^{PA} = 0.63$ and $\alpha^{PC} = 0.47$. Thus, the fraction of the immobilized component in the phosphatidylcholine spectra, $\alpha^{PC} = 0.47$, is consistent between the two independent sets of subtractions. In our previous study involving variation of the lipid/protein ratio in cytochrome oxidase-DMPC complexes (Knowles et al., 1979), it was found that there was essentially no selectivity between the phosphatidylcholine spin-label and the unlabeled phosphatidylcholine for occupation of the motionally restricted sites. Hence, the fraction of immobilized component, $\alpha^{PC} = 0.47$, corresponds to a value for the number of first shell phosphatidylcholine molecules of $n_1^{PC} = 44$, in fair agreement with the value of 55 ± 5 obtained previously from the lipid/protein titration. [Note that these complexes have a fairly low lipid/protein ratio, for which it was previously found that the relative amount of the immobilized component began to deviate from a fixed stoichiometry with respect to the protein (Knowles et al., 1979).] It appears that there may be some interaction broadening of the cardiolipin spectrum in Figure 2a. However, it seems that this does not have a marked effect on the quantitation of the spectral subtractions, since the same value is obtained for α^{PC} from the independent set of subtractions with the phosphatidic acid label.

The selectivity of the first-shell lipids for cardiolipin and phosphatidic acid can be interpreted in terms of an exchange

² Let S^A and S^B refer to the spectra from label A and label B, respectively, and let s_f and s_b refer similarly to the fluid and immobilized components. Then, with the assumption that the spectra S^A , S^B , s_f , and s_b are all normalized to the same integrated intensity, the relative compositions of the two spectra are

$$S^A = \alpha^A s_b + (1 - \alpha^A) s_f$$

$$S^B = \alpha^B s_b + (1 - \alpha^B) s_f$$

The immobilized end point of subtracting S^B and S^A is then given by

$$S^A - [(1 - \alpha^A)/(1 - \alpha^B)] S^B = [(\alpha^A - \alpha^B)/(1 - \alpha^B)] s_b$$

and the fluid end point of subtracting S^A from S^B is given by

$$S^B - (\alpha^B/\alpha^A) S^A = [(\alpha^A - \alpha^B)/\alpha^A] s_f$$

The expressions for the fractions, f , of immobilized component then follow immediately.

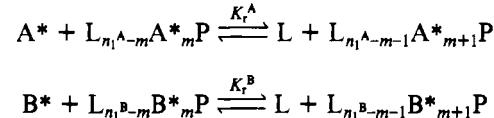
Table I: Head-Group Selectivity for the Motionally Restricted Lipids in Cytochrome Oxidase-Dimyristoylphosphatidylcholine Complexes^a

L	K_r^L/K_r^{PC}	ΔG°_{L-PC}		n_1^L	n_1^{PC}
		(cal/mol)			
CL*	5.4	1020	77	43	
PA*	1.9	390	59	44	
PS*, PG*, and PE*	1.0	0	b	b	

^a K_r , relative association constant; n_1 , first-shell occupancy.

^b $n_1^L = n_1^{PC}$.

equilibrium (Griffith & Jost, 1979; Knowles et al., 1979) between the labeled lipids, A*, B*, etc., and the unlabeled lipids, L, occupying n_1 independent sites on the protein, P:



where K_r is the relative association constant of the labeled lipid compared with that of the unlabeled lipid. The association of the spin-label with the fluid lipid component (n_f^* lipids/protein) and the immobilized lipid component (n_b^* lipids/protein) can then be expressed in terms of the following effective binding equation (Griffith & Jost, 1979; Knowles et al., 1979):

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

where the ratio n_f^* / n_b^* refers to the spin-label and is obtained from double integration of the component spectra, and n_t is the total lipid/protein mole ratio which refers essentially to the unlabeled lipid and is obtained by chemical analysis of the sample. In our previous study, it was found that $K_r^{PC} \approx 1$, implying no selectivity between the PC label and unlabeled PC in cytochrome oxidase-DMPC complexes (Knowles et al., 1979).

Within the framework of this model, selectivity can arise from a difference in the relative association constants and/or a difference in the number of sites for a particular lipid. Then one has the two extreme possibilities: either the effective number of sites is the same for the two different labels, $n_1^A = n_1^B$, or the two effective association constants are equal, $K_r^A = K_r^B$. If $n_1^A = n_1^B$, then the ratio of the two effective binding constants, K_r^A / K_r^B , can be obtained from the two measurements on complexes with the same lipid/protein ratio, n_t . From eq 1, one then obtains

$$\frac{(n_f^* / n_b^*)^A}{(n_f^* / n_b^*)^B} = \frac{K_r^B}{K_r^A} = \frac{K^B}{K^A} \quad (2)$$

where in terms of the results from the intersubtractions $(n_f^* / n_b^*)^A = (1 - \alpha^A)/\alpha^A$ and $(n_f^* / n_b^*)^B = (1 - \alpha^B)/\alpha^B$. The difference in the effective free energy of association of the two lipids with the first-shell sites on the protein is then simply given by

$$\Delta G^\circ_A - \Delta G^\circ_B = -RT \ln (K^A / K^B) \quad (3)$$

The results of the analysis of the spectra of Figure 2 in this manner, using intersubtractions, are given in Table I.

For the situation of different numbers of sites, there are two possibilities. Either there are additional sites, for which one of the labels can compete with the host lipid and the other cannot, or there are specific sites which are available to only one of the labels and not to the host lipid. In the first case,

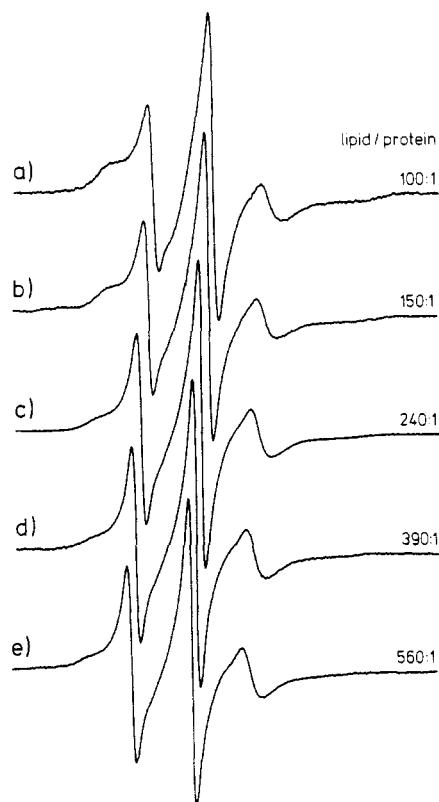


FIGURE 5: ESR spectra of the cardiolipin spin-label, 14-CLSL, in cytochrome oxidase-dimyristoylphosphatidylcholine complexes of various lipid/protein mole ratios, $T = 32^\circ\text{C}$. Total scan width = 100 G.

if it is assumed that $K_r^A = K_r^B$ in eq 1, one gets $(n_f^*/n_b^*)^A/(n_f^*/n_b^*)^B = (n_t/n_1^A - 1)/(n_t/n_1^B - 1)$, which is not soluble unless one assumes a value for, e.g., n_1^B , then a value for n_1^A may be determined. In the case of phosphatidylcholine being chosen as the "B" lipid, since $K_r^{\text{PC}} \approx 1$, this assumption reduces to the simple situation

$$n_1^A = n_t/[1 + (n_f^*/n_b^*)^A] = n_t\alpha^A \quad (4)$$

and similarly for phosphatidylcholine. This essentially represents the conservation of mass, since all relative association constants are assumed to be unity and hence for the unlabeled lipid $n_f/n_b = n_f^*/n_b^*$. The data of Figure 2 are analyzed according to this model in Table I. In the second situation, it is assumed that there are n_0^A specific sites which are available only to label A and not to label B or the host lipid and that the remaining n_1 sites are available to all lipids with no selectivity between A and B. Since the specific sites are always occupied by n_0^A molecules of label A from eq 1 for $K_r^A = K_r^B$, we get $(n_f^*/n_b^* - n_0^A)^A = (n_f^*/n_b^*)^B$, and hence, $(n_0^A/n_b^*)^A = [(n_f^*/n_b^*)^B - (n_f^*/n_b^*)^A]/(n_f^*/n_b^*)^B$, which gives the fraction of immobilized A* molecules which occupy specific sites. Comparing with phosphatidylcholine as the B lipid, this corresponds to 80% of the immobilized cardiolipid, and 50% of the immobilized phosphatidic acid, occupying specific sites.

Experiments with the various spin-labels in aliquots of a similar cytochrome oxidase-dimyristoylphosphatidylcholine sample of lipid/protein ratio 90:1 have confirmed the selectivity of phosphatidic acid relative to phosphatidylcholine and the lack of selectivity between phosphatidylethanolamine and phosphatidylcholine. Intersubtractions between the spectra from the 14-PESL and 14-PCSL spin-labels at 32°C showed them to be almost identical, implying that $K_r^{\text{PE}}/K_r^{\text{PC}} = 1$ or $n_1^{\text{PE}} = n_1^{\text{PC}}$, as found previously. Intersubtractions between the spectra of the 14-PASL and 14-PCSL spin-labels at 32°C

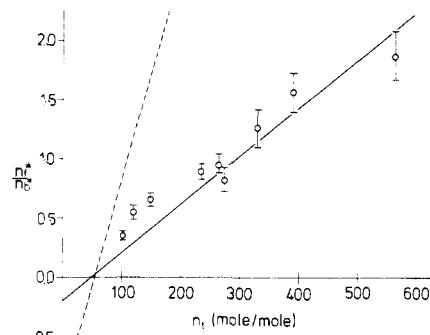


FIGURE 6: Lipid/protein titration of cytochrome oxidase-dimyristoylphosphatidylcholine complexes from the ESR difference spectra of the 14-CLSL cardiolipin spin-label. n_f^*/n_b^* is the ratio of the double-integrated intensity of the fluid and motionally restricted components in the ESR spectra of the type shown in Figure 5. n_t is the total lipid/protein mole ratio in the complex. The straight line is the predicted dependence with simple competition for $n_1 = 50$ sites and a relative association constant of $K_r = 5$. The dashed line is for $n_1 = 55$ and $K_r = 1$, which was previously used to fit the data for the 14-PCSL phosphatidylcholine spin-label (Knowles et al., 1979).

$^\circ\text{C}$ yielded $\alpha^{\text{PA}} = 0.81$ and $\alpha^{\text{PC}} = 0.67$, implying $K_r^{\text{PA}}/K_r^{\text{PC}} = 2.1$, in reasonable agreement with Table I, or, alternatively, $n_1^{\text{PA}} = 74$ and $n_1^{\text{PC}} = 61$, which are higher values than those in Table I but show the same specificity.

The ESR spectra of the cardiolipin spin-label, 14-CLSL, in cytochrome oxidase-dimyristoylphosphatidylcholine complexes of various lipid/protein ratios, at 32°C , are given in Figure 5. Comparison of these spectra with those previously published for the phosphatidylcholine spin-label in cytochrome oxidase-dimyristoylphosphatidylcholine complexes (Knowles et al., 1979) clearly shows a selectivity of cytochrome oxidase for cardiolipin over phosphatidylcholine. The spectra of Figure 5 are all composed of an immobilized spin-label component in addition to the normal fluid bilayer component. The proportion of the latter increases with increasing lipid/protein ratio in the complex, but much less rapidly than was observed previously for the phosphatidylcholine label. With the 14-PCSL label, the motionally restricted component was barely detectable at the highest lipid/protein ratios of Figure 5.

The proportions of fluid and immobilized components in the cardiolipin spectra from the complexes of the different lipid/protein ratios have been analyzed by spectral subtraction as done previously for the phosphatidylcholine spin-label (Knowles et al., 1979). The immobilized component was subtracted from the spectra of the complex to yield a fluid difference spectrum. A spectrum of the 14-PCSL label in dimyristoylphosphatidylcholine bilayer vesicles at 4°C , and with a suitably adjusted outer splitting, was used as the "immobilized" component for the subtractions. The resulting values of the lipid/protein titration for the cardiolipin label are plotted according to eq 1 in Figure 6. The experimental points do not all fall on a single straight line, indicating that the situation may be more complex than the simple exchange equilibrium of eq 1. However, the data do show a consistently higher specificity for cardiolipin than for phosphatidylcholine at all lipid/protein ratios. In addition, duplicate aliquots of some of the samples of Figure 6 were labeled with one or another of the various labels. Whenever this was done, it was invariably found that the cardiolipin label showed a higher specificity. The data at the higher lipid/protein ratios in Figure 6 (for which any effects of protein aggregation should be minimized) are consistent with a value of $K_r^{\text{CL}} = 5$ and $n_1^{\text{CL}} = 50$. The value for the relative association constant is in agreement with the independent value in Table I, and the value for the number of association sites correlates well with the

value of $n_1^{\text{PC}} = 55 \pm 5$ obtained previously for phosphatidylcholine (Knowles et al., 1979).

Discussion

The results of Figure 2 clearly indicate a selectivity for some phospholipids, namely, phosphatidic acid and cardiolipin, in the interaction of first-shell lipids with cytochrome oxidase.³ The selectivity for cardiolipin is further substantiated by the results of Figures 5 and 6. A selectivity based on charge has previously been observed with single-chain lipid spin-labels interacting with the (Na⁺,K⁺)-ATPase (Brotherus et al., 1980). In this latter case, the selectivity was predominantly of electrostatic origin, since it could be screened by high salt concentration. The situation of spin-labeled phospholipids interacting with cytochrome oxidase is somewhat more complex since no difference is detected between the zwitterionic phospholipids (PC and PE) and the singly negatively charged phospholipids (PG and PS). In addition, differential effects are observed between phosphatidic acid and cardiolipin which formally bear the same charge.⁴ Indeed, any electrostatic contribution to the lipid selectivity is expected to be small in the present work since the experiments were conducted in high ionic strength (1.0 M KCl). Studies on rod outer segment disk membranes have indicated relatively little specificity in the interaction of first-shell lipids with rhodopsin (Watts et al., 1979). This is paralleled by the lack of selectivity between many of the phospholipids (PC, PE, PS, and PG) and cytochrome oxidase, suggesting that the primary feature of the motionally restricted spin-label component is simply occupancy of first-shell sites rather than any specific interaction with the protein.

The selectivities observed for certain phospholipids thus imply some specific interaction over and above that of mere occupancy. Of particular interest is the selectivity for cardiolipin, since this both has a rather different molecular geometry from the other phospholipids and in mammalian systems, at least, seems to be somewhat specific to the inner mitochondrial membrane (Rouser et al., 1968). Although it was previously shown that cardiolipin was not essential for the oxidative activity of yeast cytochrome oxidase (Watts et al., 1978), it cannot be excluded that cardiolipin is required for respiratory control or other vectorial functions of the membrane-bound enzyme. In addition, it has also been asserted that cardiolipin is essential for the activity of the beef heart enzyme (Awasthi et al., 1971; Robinson et al., 1980). Evidence for a partial specificity for cardiolipin has also come from other spin-label studies. Titrations on a different cytochrome oxi-

³ Meierovitch & Freed (1980) have recently suggested that the spectra of lipid labels in some lipid-protein systems may be accounted for by an increase in the microscopic ordering of a single mobile species. The spectra reported here resemble neither the experimental nor the simulated spectra of these authors. Comparison of the spectral line shapes in Figures 2 and 5 with the single-component line shapes of the pure lipids in Figure 3 strongly suggests that the former are composed of more than a single component. This interpretation is further supported by spectral subtraction and by the dependence of the spectra on the lipid/protein ratio and on the phospholipid spin-label head group.

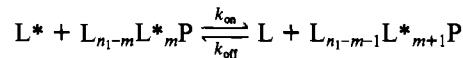
⁴ The experiments were carried out at pH 7, which is below the effective buffering range of Tris against decreases in pH. However, the quantity of negatively charged lipid spin-labels added was very small, and the samples were washed with excess buffer after labeling. Therefore, it is unlikely that the pH of the sample was significantly shifted by addition of the lipid spin-labels. The ionization of glycerol phosphate to its doubly charged state takes place at $pK \approx 6$. There will be no electrostatic enhancement of the pK of the phosphatidic acid label in a dimyristoylphosphatidylcholine host at the surface concentrations used here. Thus, it is concluded that the 14-PASL spin-label will be in its doubly charged state at pH 7.

dase-phosphatidylcholine system (Powell & Cable, 1980) have indicated a specificity in terms of an increased affinity rather than a greater number of sites. This is in broad agreement with the results of Figure 6, although the much larger range of lipid/protein ratios studied here indicates that the situation is more complex than the simple exchange model suggests. Experiments involving reconstitution of cytochrome oxidase in which unlabeled cardiolipin is mixed with phosphatidylcholine as the reconstituting host lipid have also revealed evidence for a competitive specificity (Jost & Griffith, 1978b).

Recently, Cable & Powell (1980) have suggested that an increased motionally restricted component could be observed for cardiolipin, purely as a result of the bifunctional nature of the cardiolipin molecule, even if the intrinsic association constant is $K_r = 1$, as for phosphatidylcholine. The assumption is that there is still a strong motional restriction of the spin-label chain when it occupies a second-shell position relative to the protein, if the other half of the cardiolipin molecule is in the first shell. We have previously shown for freely diffusible labels that the second-shell lipids of cytochrome oxidase are considerably less motionally restricted than those of the first shell, resembling much more the normal fluid lipids (Knowles et al., 1979). Devaux et al. (1981) have also shown, for a lipid chain *covalently attached* to rhodopsin, that the second-shell lipids have high mobility. In addition, we have recently shown that there is no selectivity for cardiolipin in rod outer segment membranes (Marsh et al., 1982). For these reasons, we conclude that the "second-shell effect" does not contribute to the selectivity for cardiolipin. The maximum specificity this effect would predict corresponds to $K_r = 2$, whereas we observe considerably higher affinities for cytochrome oxidase.

If it is assumed that the cytochrome oxidase selectivities arise from a difference in effective association constants, the data of Table I indicate that cardiolipin has a more than 5-fold greater effective affinity for the first-shell-immobilized lipid sites than phosphatidylcholine, and phosphatidic acid has an approximately 2-fold greater affinity. The increase in the effective free energy of association is relatively modest and does not imply a highly specific form of interaction; for instance, the electrostatic interaction energy between two elementary electric charges 5 Å apart in a medium of dielectric constant $\epsilon = 80$ (water) is ~ 830 cal/mol. An interpretation of the selectivity in terms of different numbers of sites seems less probable. It is unlikely, for example, that there would be extra sites for which the cardiolipin label could compete with the unlabeled phosphatidylcholine, but which were nonetheless totally inaccessible to labeled phosphatidylcholine, especially since the number of these sites (~ 35) is so large. Similarly, it is improbable that there are such a large number of specific sites for cardiolipin. A hybrid situation is, of course, possible, with some sites specific and others nonspecific but for which cardiolipin has a greater affinity.

It is interesting to note that differential affinities of the first-shell lipids also imply differential rates of exchange between the first-shell lipids in contact with the protein and the lipids in the subsequent shells away from the protein. If in the exchange equilibrium



it is assumed that the on-rate constant is the same for all species (i.e., diffusion controlled), then a relative equilibrium association constant, $K_r = k_{\text{on}}/k_{\text{off}}$, greater than 1 implies a slower off-rate constant. Thus, the data of Table I indicate that the exchange rates ($\nu_{\text{ex}} = k_{\text{off}}$) are 5 and 2 times as slow

for cardiolipin and phosphatidic acid, respectively, as that for phosphatidylcholine. These differential rates, and also the differential lipid specificities, are difficult to reconcile with the suggestion that the immobilized spin-label lipid component arises from molecules trapped between protein aggregates (Swanson et al., 1980). Indeed, it has previously been shown that trapped spin-labeled lipid has rather different spectral characteristics (Marsh et al., 1978).

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Structural Requirements for the Binding of Oligosaccharides and Glycopeptides to Immobilized Wheat Germ Agglutinin[†]

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ABSTRACT: The structural requirements for the interaction of asparagine-linked oligosaccharide moieties of glycoproteins with wheat germ agglutinin (WGA) were investigated by using affinity chromatography on a WGA-Sepharose column. So-called hybrid-type glycopeptides obtained from ovalbumin [Yamashita, K., Tachibana, Y., & Kobata, A. (1978) *J. Biol. Chem.* 253, 3862-3869] were found to have high affinity for WGA-Sepharose, whereas high mannose-type and complex-type glycopeptides were shown to have low affinity. The elution profiles of various glycopeptides modified by glycosidase treatment, Smith periodate degradation, acetolysis, and

hydrazinolysis showed that the GlcNAc β 1-4Man β 1-4GlcNAc β 1-4GlcNAc structure was essential for the binding of glycopeptides to a WGA-Sepharose column. Thus, it was revealed that both the *N,N'*-diacetylchitobiose moiety and the β -N-acetylglucosaminyl residue linked to C-4 of the β -linked mannose residue contributed to the interaction of the glycopeptide with WGA-Sepharose. The substitution at C-6 of the innermost β -N-acetylglucosaminyl residue by an α -fucosyl residue or at C-6 of the β -linked mannose residue by another mannose residue in the above structure reduced the affinity of glycopeptides for the column.

Wheat germ agglutinin (WGA)¹ has become an important tool for the isolation and characterization of a variety of glycoconjugates, especially glycoproteins. The binding spe-

cificity of WGA has been investigated in several laboratories. N-Acetylglucosamine and its β (1-4) oligomers have been found to be potent inhibitors of WGA in agglutination (Burger & Goldberg, 1967; Allen et al., 1973; Krug et al., 1973; Lotan et al., 1975), in mitogenic stimulation of lymphocytes (Brown

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¹Abbreviations used: WGA, wheat germ agglutinin; OA, ovalbumin; UA, porcine thyroglobulin unit A glycopeptide; UB, porcine thyroglobulin unit B glycopeptide.