Spin-Label Studies on the Specificity of Interaction of Cardiolipin with Beef Heart Cytochrome Oxidase†

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ABSTRACT: The selectivity of interaction of various cardiolipin analogues with beef heart cytochrome oxidase in reconstituted complexes with dimyristoylphosphatidylcholine has been studied by electron spin resonance spectroscopy, using lipids spin-labeled in the acyl chains. No difference in selectivity is observed between cardiolipin and its monolyso derivative, and similarly no selectivity is observed between phosphatidylcholine and lysophosphatidylcholine. Removal of the cardiolipin charge by methylation of the phosphate groups reduces but does not eliminate selectivity relative to phosphatidylcholine. The dependence of the lipid selectivity on head group and chain composition is in the order cardiolipin ≈ monolysocardiolipin > acylcardiolipin > dimethylcardiolipin > phosphatidylcholine ≈ lysophosphatidylcholine, where acylcardiolipin has the spin-label chain attached at the center –OH of the head group. The degree of association of the negatively charged cardiolipin derivatives with cytochrome oxidase decreases with increasing salt concentration, to a level comparable to that for dimethylcardiolipin. At high ionic strength there is still a marked selectivity relative to phosphatidylcholine. Li^+ ions are more effective in screening the interaction than are Na^+ ions, and divalent ions are more effective than monovalent ions. The selectivity for cardiolipin is only slightly reduced on titrating the protein to high pH. Alkylation of the protein with *N*-ethylmaleimide has little effect on the titration behavior. Covalent modification of the protein by reaction with citraconic anhydride decreases the selectivity of interaction with cardiolipin. It is concluded that cardiolipin possesses an additional specificity of interaction with cytochrome oxidase other than that of purely electrostatic origin.

In previous spin-label studies of lipid–protein interactions with cytochrome oxidase it has been found that approximately 50 lipids per 200 000-dalton protein undergo motional restriction as a result of direct association with the intramembranous surface of the protein (Jost et al., 1973; Knowles et al., 1979). On the basis of currently available structural data, this corresponds approximately to the number of lipids that may be accommodated in a single shell around the intramembranous perimeter of the protein [see Marsh (1985) for a review]. A lipid head group specificity has also been demonstrated for the association of the spin-labeled lipids in this protein-interacting component (Cable & Powell, 1980; Knowles et al., 1981; Marsh et al., 1982). The relative association constants were found to lie in the order cardiolipin > phosphatidic acid ≈ stearic acid > phosphatidylserine ≈ phosphatidylglycerol ≈ phosphatidylcholine ≈ phosphatidylethanolamine. Particularly interesting is the specificity for cardiolipin, a lipid that in mammalian systems is unique to the inner mitochondrial membrane and that has been found to copurify with cytochrome oxidase and to be particularly effective in its reconstitution (Awasthi et al., 1971; Yu et al., 1975; Robinson et al., 1980).

In the present study we have investigated the origin of the specificity of interaction of cardiolipin with beef heart cytochrome oxidase. We have examined the effects of modification of the structure of the cardiolipin molecule and chemical modification of the protein, as well as the salt and pH dependence of the interaction. It is concluded that direct electrostatic effects are only partially responsible for the observed selectivity of cytochrome oxidase for cardiolipin. The lipid–protein interactions of the different cardiolipin analogues are of additional interest, since it has been found that not all of these various lipids form lamellar phases when dispersed in water and that these nonlamellar phase transformations can be triggered by changes in salt concentration (Powell & Marsh, 1985).

Materials and Methods

Dimyristoylphosphatidylcholine (DMPC)[1] puriss was obtained from Fluka (Buchs, Switzerland). Cytochrome *c* (grade VI) was from Sigma (St. Louis, MO). The spin-labeled stearic acid, 14-SASL, was synthesized essentially by the method of Hubbell and McConnell (1971). Spin-labeled phosphatidylcholine, 14-PCSL, was synthesized from 14-SASL, 1,1′-carbonyldiimidazole, and egg lysophosphatidylcholine from

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[1] Abbreviations: 14-SASL, 2-(12-carboxydodecyl)-2-butyl-4,4-dimethyl-3-oxazolidinyloxy; 14-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-*O*-[13-(2-butyl-4,4-dimethyl-3-oxy-2-oxazolidinyl)tridecanoyl]-*sn*-glycero(3)phospho]-*sn*-glycerol; 14-lyso-CLSL, 1-[1-acyl-2-lyso-*sn*-glycero(3)phospho]-3-[1-acyl-2-*O*-[13-(2-butyl-4,4-dimethyl-3-oxy-2-oxazolidinyl)tridecanoyl]-*sn*-glycero(3)phospho]-*sn*-glycerol; 14-di-MeCLSL, 1-[1,2-diacyl-*sn*-glycero(3)-*O*-methylphospho]-3-[1-acyl-2-*O*-[13-(2-butyl-4,4-dimethyl-3-oxy-2-oxazolidinyl)tridecanoyl]-*sn*-glycero(3)-*O*-methylphospho]-*sn*-glycerol; 14-acyl-SLCL, 1-(3-*sn*-phosphatidyl)-2-*O*-[13-(2-butyl-4,4-dimethyl-3-oxy-2-oxazolidinyl)tridecanoyl]-3-(3-*sn*-phosphatidyl)-*sn*-glycerol; 14-PCSL, 1-acyl-2-*O*-[13-(2-butyl-4,4-dimethyl-3-oxy-2-oxazolidinyl)tridecanoyl]-*sn*-glycero(3)-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero(3)phosphocholine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; TLC, thin-layer chromatography; KP_i, potassium phosphate; SDS, sodium dodecyl sulfate.

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Lipid Products (South Nutfield, U.K.) according to the method of Boss et al. (1975). Spin-labeled cardiolipin, 14-CLSL, was prepared from 14-SASL and monolysocardiolipin (Avanti Biochemicals, Inc., Birmingham, AL), according to the above acylation method as described by Cable and Powell (1980). All preparative chromatography and handling of this and other cardiolipin analogues were done under a protective atmosphere of Ar or N_2. The lipid spin-labels were dissolved in ethanol at a concentration determined by phosphate analysis (Eibl & Lands, 1969) and stored at −30 °C.

14-lyso-CLSL was prepared by a similar procedure from dilysocardiolipin. Beef cardiolipin purchased from Sigma Chemical Co. (St. Louis, MO) was hydrolyzed by using phospholipase A_2 from pig pancreas (Boehringer Mannheim GmbH, Mannheim, FRG), following the technique of De Haas et al. (1968). Dilysocardiolipin was isolated by extraction (Bligh & Dyer, 1959) and then by chromatography on silica gel (Merck 60, reinst, 70–230 mesh) from E. Merck (Darmstadt, FRG) in a 2.8 × 20 cm column, eluting sequentially with ~500 mL each of $CHCl_3/CH_3OH/H_2O$ (100:15:1 v/v), $CHCl_3/CH_3OH/H_2O$ (65:30:3 v/v), and then $CHCl_3/CH_3OH/H_2O$ (65:35:5 v/v). The fractions containing dilysocardiolipin were separated by TLC on silica gel 60 F-254 from Merck, using a solvent system of $(CH_3)_2CO/CHCl_3/CH_3OH/CH_3COOH/H_2O$ (40:30:10:10:5 v/v) and detecting lipid phosphorus with molybdenum blue spray (Applied Science Labs, State College, PA). The fractions containing dilysocardiolipin (R_f = 0.23) were pooled and purified to homogeneity by preparative TLC on 2-mm layers as above. The homogeneous dilysocardiolipin (33.6 mg) was acylated with a 20% excess of 1,1′-carbonyldiimidazole and 14-SASL. 14-lyso-CLSL was isolated by preparative TLC as the component with the same migration as authentic monolysocardiolipin (R_f = 0.50) in the solvent system used to isolate dilysocardiolipin. The yield from dilysocardiolipin after purification was 7.5% on the basis of recovery of lipid phosphorus (Eibl & Lands, 1969).

14-diMeCLSL was prepared from 14-CLSL using diazomethane by the method of Gwak (1983). 14-CLSL (0.5 μmol) dissolved in 200 μL of $CHCl_3$, 400 μL of CH_3OH, and 200 μL of 0.1 M HCl at 0 °C was extracted by adding 200 μL of $CHCl_3$ and 200 μL of 0.1 M HCl and separating the $CHCl_3$ layer by centrifugation. The 14-CLSL was thereby converted from the sodium salt to the free acid. Then 2.5 equiv of CH_2N_2 were added to the 14-CLSL in $CHCl_3$ at 0 °C and allowed to stand for 15 min. If the yellow color from excess CH_2N_2 was not apparent at the end of this time, small aliquots of CH_2N_2 were added until the color persisted. The solvents and excess reagent were then removed in a hood under a stream of N_2. The conversion was quantitative as judged by TLC in the above solvent system (R_f = 0.95). The residue was dissolved in $CHCl_3$ and then redried by using N_2 and finally taken up at the desired concentration in ethanol. This product had an R_f = 0.66, running much faster than cardiolipin (R_f = 0.04), in $CHCl_3/CH_3OH/NH_4OH$ (concentrated) (65:35:4 v/v), as expected for the less polar ester. This product was readily separable from fatty acids in hexane/ether (1:1 v/v). 14-diMeCLSL was stable to storage in ethanol or $CHCl_3$ for months as judged by TLC with detection by fluorescence quenching and staining with I_2 vapor. The product did not react with molybdate blue reagent at room temperature but gave the expected phosphate content after digestion and assay according to Eibl and Lands (1969).

14-acyl-SLCL was prepared by acylation of beef cardiolipin (Sigma Chemical Co.) according to the method of Fowler (1983). This lipid was carefully dried at room temperature in vacuo, resuspended in dry $CHCl_3$ (distilled from P_2O_5), and taken to dryness again. This process was repeated several times, and then the dry lipid was held in vacuo over P_2O_5 overnight. The lipid was then taken up in dry $CHCl_3$ and transferred into a $CHCl_3$ solution of the symmetrical anhydride of 14-SASL (Selinger & Lapidot, 1966) and 4-(dimethylamino)pyridine obtained from Fluka (Buchs, Switzerland) (recrystallized). One mole of the 14-SASL anhydride and 1 mol of 4-(dimethylamino)pyridine were used per mole of cardiolipin. The mixture was magnetically stirred in a closed container at room temperature for 3 days and the product isolated by preparative TLC on silica gel 60 F-254, 2 mm in thickness, with $CHCl_3/CH_3OH/NH_4OH$ (concentrated) (65:15:1 v/v) (R_f = 0.80). In a solvent system of $CHCl_3/CH_3OH/NH_4OH$ (concentrated) (65:35:4 v/v) this lipid also had a faster migration rate (R_f = 0.17) than cardiolipin (R_f = 0.04) on silica gel 60 F-254, 0.25-mm layers. The overall yield of final product on the basis of recovery of lipid phosphate was 20%.

Cytochrome *c* oxidase was prepared from beef heart by the method of Yu et al. (1975) with the modifications given by Cable and Powell (1980). The enzyme was stored and transported at 190 K or in N_2(l). Lipid substitution by DMPC was carried out according to the method of Knowles et al. (1981), with omission of sucrose and KCl from the buffers. The lipid content after substitution was characterized as described earlier (Knowles et al., 1979). Enzyme activity was assayed at 25 °C in 40 mM KP_i (pH 6.7) by using 30 μM reduced cytochrome *c*. The time course was fitted to a first-order rate constant, using a linear least-squares routine (Yonetani & Ray, 1965). The molecular weight of the enzyme was taken as 200 000. The residual cholate levels in the reconstituted enzyme were assayed using [^{14}C]cholate and found to be less than 1 mol/mol of protein, or approximately 1 cholate/100 lipid molecules. The reconstituted, membranous cytochrome oxidase was suspended at a concentration of 1 mg/mL in 10 mM Tris·HCl and 1 mM EDTA·Na_2 (pH 7.4) and labeled by adding 5 μL of the spin-labeled lipid in ethanol to 1 mL of enzyme suspension. After incubation at 25 °C for approximately 1 h, the enzyme was diluted to 10 mL, with buffer of the desired pH or salt concentration, and then centrifuged at 80000*g* for 25 min in a fixed-angle 65 rotor from Beckman Instruments Co. The pellet was then resuspended in buffer of the same required pH or salt concentration and centrifuged again. Finally, the pellet was transferred to a 1 mm diameter capillary, which was centrifuged at full speed on a benchtop centrifuge to pack the pellet, the excess supernatant was removed, and the sample was then sealed.

N-Ethylmaleimide and citraconic anhydride were from Sigma, St. Louis, MO. Reaction with cytochrome oxidase was carried out by adding an excess of the reagent, either neat or in a small volume of ethanol, to the DMPC-substituted enzyme in buffer. Alkylation with *N*-ethylmaleimide was performed in 10 mM phosphate buffer, pH 7.4, and 0.1 M sodium borate buffer, pH 8.5, was used for the reaction with citraconic anhydride. In each case, the reaction was terminated by adding an excess of 0.1 M Tris, pH 8.0 buffer. Available amino groups on the protein were assayed by colorimetric reaction with trinitrobenzenesulfonate (TNBS), as described in Fields (1972).

ESR spectra were recorded on a Varian E-12 9-GHz Century Line spectrometer equipped with nitrogen gas flow temperature regulation. The samples, in sealed capillaries, were contained in the standard 4 mm diameter quartz tubes

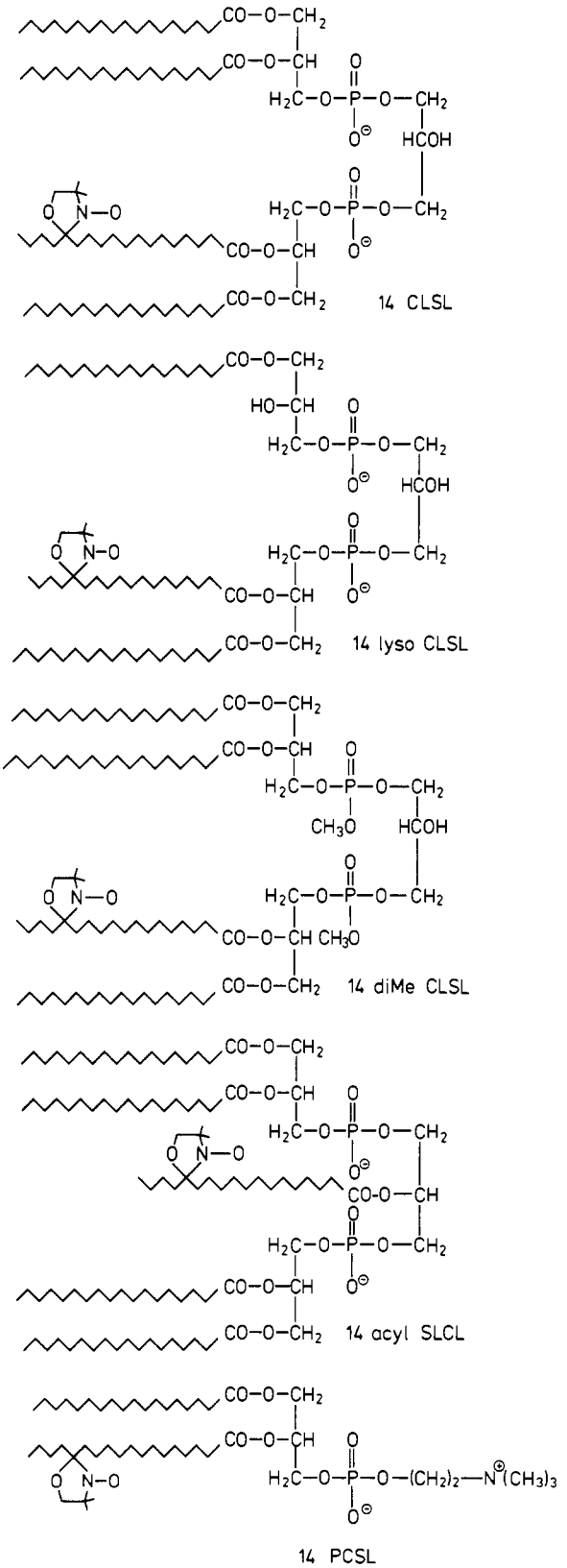

FIGURE 1: Structures of cardiolipin and phosphatidylcholine spin-label analogues. From top to bottom: cardiolipin, 14-CLSL; lysocardiolipin, 14-lyso-CLSL; dimethylcardiolipin, 14-diMeCLSL; acylcardiolipin, 14-acyl-SLCL; phosphatidylcholine, 14-PCSL.

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 with interactive graphics. Spectral subtraction was performed according to the protocols described in Marsh (1982).

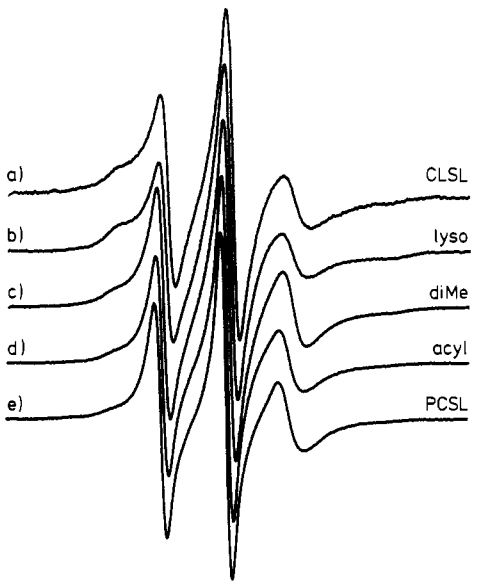

FIGURE 2: ESR spectra of cardiolipin analogues spin-labeled at the C-14 position in cytochrome oxidase/DMPC complexes of lipid/protein ratio 210:1 mol/mol: (a) cardiolipin, 14-CLSL; (b) monolysocardiolipin, 14-lyso-CLSL; (c) dimethylcardiolipin, 14-diMeCLSL; (d) acylcardiolipin, 14-acyl-SLCL; (e) phosphatidylcholine, 14-PCSL. Buffer: 10 mM Tris and 1 mM EDTA, pH 7.4. Total scan width = 100 G; T = 30 °C.

RESULTS

The structures of the different spin-labeled cardiolipin derivatives are given, together with that of spin-labeled phosphatidylcholine, in Figure 1. The ESR spectra of the various spin-labeled analogues in a cytochrome oxidase/DMPC recombinant of lipid/protein ratio 210:1 mol/mol, at 30 °C, are shown in Figure 2. As found previously (Knowles et al., 1979, 1981), the spectra consist of two components, one corresponding to normal fluid bilayer lipids and the other to more motionally restricted lipids interacting directly with the intramembranous surface of the protein. The fluid component spectrum consists of the three sharp peaks in Figure 2 and is similar to the spectrum of the lipids alone (not shown). The spectrum of the motionally restricted component is broader than that of the fluid component and is resolved in the outer wings of the latter in Figure 2. A clear selectivity for the motionally restricted component is seen between the different spin-labeled lipids in Figure 2. Both cardiolipin and monolyso-CL display a strong preferential association with the protein relative to phosphatidylcholine. That for dimethyl-CL and acyl-CL is intermediate. The relative amounts of the fluid and motionally restricted components may be determined by spectral subtraction, using the spectra of sonicated DMPC vesicles in the fluid and gel phases to approximate the individual components. From this it is possible to characterize the lipid specificity in terms of the equation for equilibrium association with the protein used previously (Brotherus et al., 1981; Knowles et al., 1979):

$$(n_f^*/n_b^*) = n_t/(N_1K_r) - 1/K_r \quad (1)$$

where $(n_f^*/n_b^*) = (1-f)/f$ is the ratio of fluid to motionally restricted spin-label components; n_t is the total lipid/protein ratio in the complex; N_1 is the total number of lipid sites directly interacting with the protein (first-shell sites); and K_r is the average association constant of the spin-label with these sites, relative to that of the background host lipid (DMPC). It was demonstrated previously that the selectivity for cardiolipin arises from an increase in average association constant, K_r, rather than in the number of sites, N_1 (Cable & Powell,

Table I: Selectivity for the Motionally Restricted Component of Spin-Labeled Cardiolipin and Phosphatidylcholine Analogues in Beef Cytochrome Oxidase/Dimyristoylphosphatidylcholine Complexes of Lipid/Protein Ratio 210:1 mol/mol[a]

L	I^b	f^c	K_r^L/K_r^{PC}	$\Delta G°_L - \Delta G°_{PC}$ (cal/mol)
CL*	0.01	0.65	3.6	−770
	3.0	0.54	2.3	−500
lyso-CL*	0.01	0.67	3.9	−830
	3.0	0.44	1.5	−250
diMeCL*	0.01	0.59	2.8	−620
	3.0	0.47	1.7	−330
acyl-CL*	0.01	0.42	1.4	−210
lyso-PC*	0.01	0.32	0.9	+50
PC*	0.01	0.34	1.0	0

[a] T = 30 °C. [b] I is the ionic strength. [c] Estimated error in f based solely on random uncertainties in spectral subtractions is ±0.02.

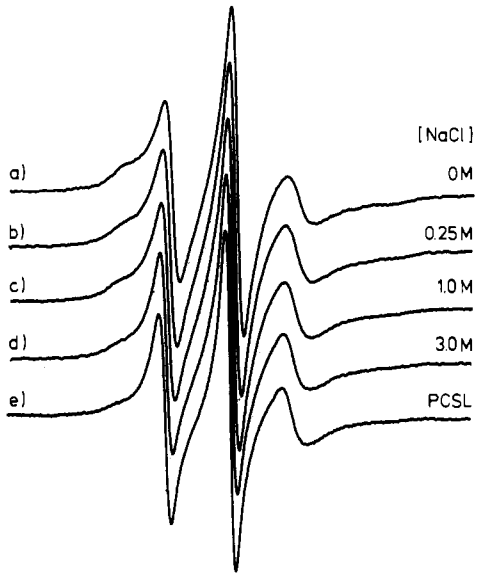

FIGURE 3: ESR spectra of spin-labeled cardiolipin, 14-CLSL, in cytochrome oxidase/DMPC complexes of lipid/protein ratio 180:1 mol/mol at different NaCl concentrations: (a) 14-CLSL with 0.0 M NaCl; (b) 14-CLSL with 0.25 M NaCl; (c) 14-CLSL with 1.0 M NaCl; (d) 14-CLSL with 3.0 M NaCl; (e) 14-PCSL with 0.0 M NaCl. Buffer: 10 mM Tris and 1 mM EDTA, pH 7.4. Total scan width = 100 G; T = 30 °C.

1980; Knowles et al., 1981). Thus, from eq 1, the selectivity relative to PC can be expressed in terms of the ratio of the respective association constants:

$$(n_f^*/n_b^*)^{PC}/(n_f^*/n_b^*) = K_r/K_r^{PC} \quad (2)$$

and the difference in effective free energy of association is given by $\Delta G° - \Delta G°_{PC} = -RT \ln (K_r/K_r^{PC})$. The fractions, f, of the motionally restricted component and selectivities of the various labels relative to PC are given in Table I. Little selectivity is seen between the parent labels and their lyso derivatives, both for cardiolipin and phosphatidylcholine. A very marked selectivity is seen for all the cardiolipin derivatives relative to phosphatidylcholine. A greater specificity is found for the charged derivatives, CL and lyso-CL, than for the uncharged derivative diMeCL, but the latter still exhibits a strong specificity relative to phosphatidylcholine. acyl-CL, although charged, apparently exhibits a lesser association than diMeCL, but in this case the spin-label is on a different chain which might not sense the protein interface so directly.

More information on the electrostatic effects is obtained from the salt dependence of the lipid–protein association. The ESR spectra of the 14-CLSL cardiolipin spin-label in a cytochrome oxidase/DMPC complex of lipid/protein ratio 180:1 mol/mol, suspended in buffers of increasing NaCl concentration, are given in Figure 3. As the salt concentration is increased from 0 to 3 M NaCl, the proportion of the motionally restricted spin-label component decreases relative to that of the fluid component. The results of spectral subtraction for a sample of lipid/protein ratio 140:1 mol/mol, in experiments in which both LiCl and NaCl concentration has been varied, are given in Figure 4. A qualitatively very similar salt dependence was obtained for the sample with lipid/protein ratio 180:1 mol/mol (data not shown). It is seem that in Figure 4 the fraction of motionally restricted 14-CLSL spin-label initially decreases rather steeply with increasing salt concentration (particularly for LiCl) and then flattens off at concentrations of 1.0–2.0 M NaCl. At this salt concentration the electrostatic contribution to the lipid–protein interaction is presumably screened by counterions. Significantly, the limiting value for the fraction of motionally restricted cardiolipin spin-label in high salt corresponds rather closely to that obtained for the uncharged diMeCL spin-label. (Note that the latter also exhibits an appreciable salt dependence—see Table I). Even in the presence of 3 M NaCl, however, the fraction of motionally restricted cardiolipin spin-label is considerably higher than that for the phosphatidylcholine spin-label, which shows relatively little salt dependence (data not shown). Lithium chloride, which has a higher solubility than NaCl, causes an additional decrease in degree of association at concentrations greater than 1 M, possibly because of changes in hydration. Even at 7 M LiCl, however, the degree of association of the cardiolipin spin-label is significantly greater than that for the phosphatidylcholine spin-label. The relative selectivities at both low and high NaCl concentration are given for the different cardiolipin analogues in Table I.

It is also of interest to examine the effect of divalent cations on the selectivity for cardiolipin. The relative association constants of spin-labeled cardiolipin for cytochrome oxidase/

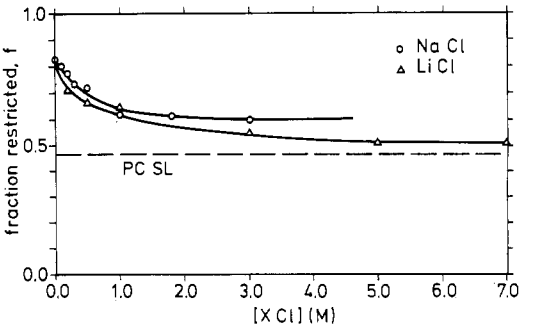

FIGURE 4: Salt dependence of the fraction of motionally restricted cardiolipin spin-label 14-CLSL in cytochrome oxidase/DMPC complexes of lipid/protein ratio 140:1 mol/mol at 30 °C: (○) 14-CLSL with NaCl; (△) 14-CLSL with LiCl; (---) phosphatidylcholine spin-label 14-PCSL. Buffer: 10 mM Tris and 1 mM EDTA, pH 7.4.

Table II: Relative Association Constants, K_r, of Spin-Labeled Cardiolipin with Cytochrome Oxidase in the Presence of 0.4 M Concentration of Mono- and Divalent Cations[a]

salt	I^b	K_r/K_r(NaCl) (exptl)	a_{CL*} (nm)	K_r/K_r(NaCl) (calcd)[c]
NaCl	0.4	1.00	1.7	1.00
LiCl	0.4	0.93	1.0	0.94
$MgCl_2$	1.2	0.79	1.7	0.78
$CaCl_2$	1.2	0.82	1.7	0.78

[a] Values are normalized to the value for NaCl, K_r(NaCl). [b] I is the ionic strength. [c] Calculated value according to Debye–Hückel theory, as described in the text.

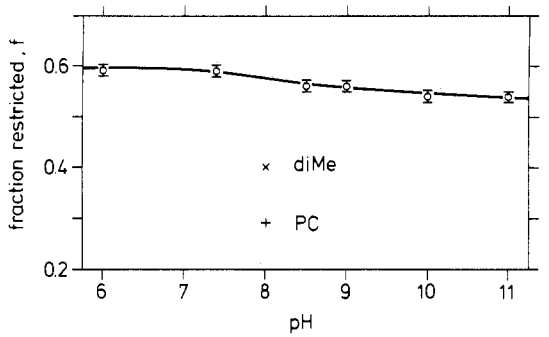

FIGURE 5: pH dependence of the fraction of motionally restricted cardiolipin spin-label 14-CLSL in cytochrome oxidase/DMPC complexes of lipid/protein ratio 220:1 mol/mol at 30 °C. Values for the dimethylcardiolipin spin-label 14-diMeCLSL (×) and for the phosphatidylcholine spin-label 14-PCSL (+) in complexes of the same lipid/protein ratio, at pH 8 and 30 °C, are given for comparison.

DMPC complexes in 0.4 M solutions of different monovalent and divalent cations are given in Table II. In order to avoid assuming a value for N_1 in eq 1, the relative association constants derived from the fractions of motionally restricted spin-label are normalized to the value $K_r(NaCl) = 1$, obtained in 0.4 M NaCl. It is seen that the relative association constants are considerably reduced, and to roughly the same extent, in the 0.4 M solutions of the divalent Mg^{2+} and Ca^{2+} ions, when compared to the values obtained in 0.4 M NaCl. This reduction can be attributed, at least in part, to the greater ionic strength of the divalent salt solutions, as indicated by the calculations using Debye–Hückel theory to be discussed later. Titrations with divalent ions (for a sample with different lipid/protein ratio) also confirmed that Ca^{2+} and Mg^{2+} ions screen more effectively than do Na^+ ions (data not shown). As already seen in Figure 4, the relative association constant is also reduced in LiCl as compared to NaCl, although this must be a more specific interaction, rather than an ionic strength effect.

The pH dependence of the cardiolipin spin-label-cytochrome oxidase interaction at low salt concentration is given in Figure 5. From this it is seen that there is a relatively small dependence of the fraction of motionally restricted 14-CLSL spin-label on pH over the range studied. The relative association constant, K_r, at pH 10 is reduced by a factor of approximately 0.8 compared with that at pH 6 or 7. The pK_a of cardiolipin is expected to lie below this pH range (Seddon et al., 1983), and thus the decrease in fraction of motionally restricted 14-CLSL from pH 7 to pH 10 is presumably attributable to titration of amino acid residues on the protein. At the highest pH's (pH 10 and 11), it is conceivable that the spin-labels could be partially hydrolyzed. However, since the decrease in f observed in Figure 5 is found to occur at pH 8–9, it is unlikely that possible hydrolysis contributes appreciably to the titration behavior. Alkylation of the protein by *N*-ethylmaleimide did not significantly affect the pH dependence of the fraction of motionally restricted 14-CLSL (data not shown).

The effect of covalent modification of the protein lysine residues on the selectivity of interaction with cardiolipin has also been studied. Reaction of the reconstituted cytochrome oxidase/DMPC complex with citraconic anhydride, which should reverse the sign of the positively charged lysine ϵ-amino groups at neutral pH, gave rise to a considerable reduction in the fraction of motionally restricted cardiolipin spin-label associated with the protein. The time dependence of the reaction on adding an approximately twofold excess (with respect to total lysine groups) of citraconic anhydride is given in Table III. Residual lysine residues available for reaction with TNBS were assayed in 1% SDS after the various incubation times. Experiments using a much larger excess of citraconic anhydride gave faster derivatization, but the residual available lysine groups remained at approximately 30% of the initial value. The total decrease in fraction of cardiolipin spin-label associated with the protein is greater than that obtained on titration to high pH and comparable to that found for dimethylcardiolipin or for cardiolipin at high salt concentrations (as judged by the changes in relative association constant).

Table III: Fraction of Motionally Restricted Spin-Labeled Cardiolipin, f, in Cytochrome Oxidase/DMPC Complexes of Lipid/Protein Ratio 150:1 mol/mol, as a Function of Time of Derivatization, t, with Citraconic Anhydride (Twofold Excess)[a]

t (min)	lysine[b] (%)	f	$K_r/K_r(0)$[c]
0 (control)	100	0.69	1.00
30	45	0.64	0.80
60	30	0.60	0.67

[a] ESR spectra taken at 30 °C. [b] Percentage of lysine groups available for reaction with TNBS in 1% SDS. [c] Relative association constant, K_r, normalized to the control value (at zero time).

Discussion

The results of the present study provide considerably more insight into the origin of the specificity previously observed in the interaction between spin-labeled cardiolipin and cytochrome oxidase (Cable & Powell, 1980; Knowles et al., 1981). This has been achieved by using spin-labeled cardiolipin analogues either with different numbers of acyl chains or in which the polar group charge has been eliminated by methylation of the phosphate groups (cf. Figure 1). The effects of screening the electrostatic interactions with various ions and of changing the protein charge by pH titration and covalent modification have also been studied. In this way, the hydrophobic contribution and the electrostatic and other head group contributions, e.g., from hydration, to the lipid–protein interactions have been distinguished.

Comparison of the results for both the cardiolipin and phosphatidylcholine spin-labels with those for the corresponding lyso derivatives (see Table I) indicates that decreasing the number of acyl chains by one has very little effect on the degree of association of either lipid. This result has several important implications. First, there is evidently no cooperative geometrical effect of the number of chains of the type suggested by Lee (1973). Second, reasoning by extrapolation, the specificity of interaction with cardiolipin relative to phosphatidylcholine does not arise from the greater number of chains per molecule for the former. Third, the contribution from the acyl chains to the energetics of lipid–protein interaction is essentially the same as that for lipid–lipid interactions. The latter can be estimated from the chain length dependence of the free energy of self-association of phospholipid molecules, which has been found to be approximately $-0.9RT$ per methylene group per chain [see, e.g., King and Marsh (1987) and Marsh and King (1986)]. Thus for chains of 18 C atoms length, the hydrophobic free energy of association between lipid and protein is approximately 9 kcal·mol^{-1} per chain, relative to a standard state of the lipid monomer in water.

Acylcardiolipin, with five chains, displays an apparently lower degree of association than does cardiolipin but nonetheless a very marked selectivity relative to phosphatidylcholine. The spin-labeled chain of acylcardiolipin is, however, not equivalent to that of the other four acyl chains of cardiolipin (cf. Figure 1). It is quite possible that this chain disturbs the head group region somewhat and also that it is unable to

associate with the protein in quite so direct a manner as do the other chains. Consequently, it might be that this chain partly samples the second lipid shell surrounding the protein, as was found for a spin-labeled chain covalently attached to rhodopsin (Davoust & Devaux, 1982). This could explain the lower intensity of the motionally restricted chain component from acyl-CLSL, relative to that obtained from the parent cardiolipin spin-label.

The head group dependence of the degree of association of the different analogues in Table I indicates that the selectivity for cardiolipin is not simply electrostatic, since the uncharged dimethyl derivative still exhibits a specificity, albeit reduced, relative to phosphatidylcholine. This is further supported by the ionic strength dependence. The ionic screening at high sodium chloride concentration reduces the extent of association, but to a level closer to that for dimethylcardiolipin than that for phosphatidylcholine (see Figure 4 and Table I). In addition, lithium chloride reduces the degree of cardiolipin association to a greater extent than does sodium chloride, suggesting that ion binding and concomitant reduction in lipid hydration may also reduce the degree of association. In this respect, it is interesting to note that high salt concentration also decreases the degree of association of the uncharged dimethyl derivative to a certain extent. Thus the preferential association of the cardiolipin analogues is not solely electrostatic and depends on the detailed head group structure and possibly also hydration. It has previously been shown that the increase in the average relative association constant K_r defined in eq 1 corresponds to a generalized specificity of cardiolipin for all $N_1 = 50$ association sites on cytochrome oxidase, rather than a higher specificity for a smaller number of sites (Powell et al., 1985). Thus it seems likely that both the electrostatic and nonelectrostatic contributions to the selectivity of the cardiolipin interaction are of a generalized nature (i.e. all association sites are similar), although a detailed comparison of spin-label to protein titrations at low and high ionic strengths has yet to be performed.

The salt dependence of the selectivity given in Figure 4 can be interpreted, at least semiquantitatively, in terms of the Debye–Hückel theory of electrolyte solutions. The relative association constant, K_r, in eq 2 is defined in terms of concentrations and is related to the true relative association constant K_r^0 via the activity coefficients γ_i of the different species:

$$K_r = K_r^0(\gamma_{L^*}\gamma_{LP}/\gamma_{L^*P}\gamma_L) \quad (3)$$

where γ_{L^*} and γ_L are the activity coefficients of the spin-labeled and unlabeled lipid, respectively, and γ_{L^*P} and γ_{LP} are similarly defined for the lipid–protein complexes. The Debye–Hückel expression for the activity coefficients is [see, e.g., Robinson and Stokes (1955)]

$$\ln \gamma_i = \frac{-Z_i^2 e^2}{8\pi\epsilon_0\epsilon kT} \frac{\kappa}{1 + \kappa a_i} \quad (4)$$

where Z_i is the charge on species i, a_i is the effective interaction distance of species i with counterions, and the inverse Debye screening length is $\kappa = (2000N_A e^2 I/\epsilon_0\epsilon kT)^{1/2}$, I being the ionic strength of the electrolyte. Hence, from eq 3 and 4, the ionic strength dependence of the relative association constant is given by

$$\ln (K_r/K_r^0) = \frac{-e^2\kappa}{8\pi\epsilon_0\epsilon kT}\left[\frac{Z_{L^*}^2}{1 + \kappa a_{L^*}} + \frac{Z_P^2}{1 + \kappa a_P} - \frac{(Z_P + Z_{L^*})^2}{1 + \kappa a_{L^*P}}\right] \quad (5)$$

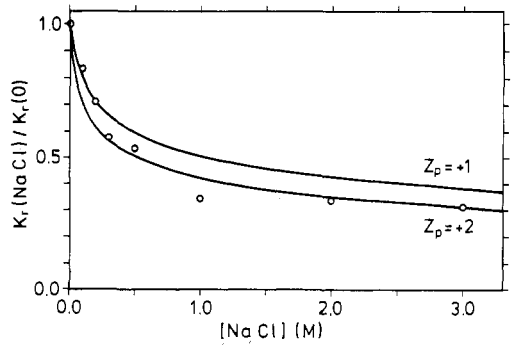

FIGURE 6: Salt-induced screening of the relative association constant of 14-CLSL cardiolipin spin-label as a function of NaCl concentration. Experimental points are deduced from the data of Figure 4 and are normalized to the value in the absence of salt (10 mM Tris, pH 7.4). The full lines are the theoretical curves deduced from Debye–Hückel theory according to eq 5, with $Z_{CL^*} = -1$, $a_{CL^*} = 0.17$ nm, $Z_P = +1$ or +2, and $a_P = 2.5$ nm. The theoretical values are normalized to ionic strength $I = 0.01$.

where L*, P, and L*P represent the lipid spin-label, the protein, and the lipid–protein complex, respectively. This expression has been fitted to the data for the 14-CLSL spin-label in sodium chloride in Figure 6. Values of $a_P = 2.5$ nm and $a_{L^*} = 0.17$ nm were assumed for the interaction radii of cytochrome oxidase and cardiolipin, respectively, with Na^+ ions. The charge per lipid phosphate was taken as $Z_{L^*} = -1$, and calculations are presented for two values of the net charge on the protein: $Z_P = +1$ and +2, respectively. With these values it is possible to represent the salt dependence of the relative association constant for cardiolipin reasonably accurately. The data from the sample with 180:1 mol/mol lipid/protein ratio (Figure 3) can be similarly fitted with slightly different values of the effective interaction radii (data not shown). A similar, qualitative fit to the ionic strength dependence of the selectivity of interaction of negatively charged lipids has also been obtained previously with data from the Na^+,K^+-ATPase (Esmann & Marsh, 1985), if the interaction distance a_{L^*} is treated as a parameter to be fitted. The enhanced screening of the interaction by divalent ions is illustrated in Table II, in which the relative effectiveness of mono- and divalent ions can be described reasonably well by the increase in ionic strength in eq 5. The more effective screening by Li^+ ions relative to Na^+ ions cannot, of course, be ascribed simply to ionic strength effects. A possible explanation lies in the smaller ionic radius of Li^+, as illustrated empirically by a reduction in a_{L^*} in Table II. It is quite likely that the relative effects of the different ions on lipid hydration also play a significant role in reducing the lipid selectivity. It should also be noted that the Debye–Hückel theory is used principally as an empirical method of quantitating the ionic screening effects. This is the simplest electrostatic description, which strictly speaking applies only to dilute solutions. However, it has long been realized that the formulation can be used to fit the behavior of more concentrated electrolyte solutions, if a_i is treated as an adjustable parameter (Robinson & Stokes, 1955). The present data do not justify a more complicated treatment.

The pH dependence in Figure 5 provides interesting insights into the specificity of interaction with the positively charged amino acid residues of the protein. Since the pK_a of the phosphate groups of cardiolipin lies outside the range investigated, any change in lipid association must be attributed to titration of the protein. A modest decrease is observed in the high-pH region, presumably arising from titration of the lysine and/or histidine side chains, but this change is still smaller than the decrease induced by salt. Apparently, positively

charged residues with even greater pK_a's must be involved in the selectivity of the lipid–protein interactions.

In contrast, chemical modification using a reagent (citraconic anhydride) which actually reverses the sign of the electrostatic charge on the lysine groups gives rise to a considerably larger decrease in the fraction of spin-labeled cardiolipin associated with the protein, comparable to that found in high salt (Table III). This gives a clear indication of the involvement of the lysine side chains in the specificity of cardiolipin interaction with cytochrome oxidase. Exact quantitation has proved difficult, and it is probable that there are quite distinct populations of lysine groups which exhibit differential interactions with the surrounding lipid molecules. This topic deserves further study, correlated with attempts to achieve selective modification of the different groups of basic residues.

Of particular relevance to the present work are the recent studies by McMillen et al. (1986), who have labeled cytochrome oxidase using negatively charged phospholipids with a benzaldehyde moiety attached to the polar head group. In this way they were able to identify lysine residues on the native enzyme that are situated at the polar–apolar interface of the membrane. Subunits III and V–VII were found to bear lysines involved in interactions with the lipid head groups. At least some of these residues must be implicated in the selectivity of interaction with the cardiolipin analogues studied here.

It is also interesting to compare the present results with those on the pH and salt dependence of the enzymatic reaction with cytochrome *c* (Sinjorgo et al., 1986; Brzezinski & Malström, 1986) and the effects of covalent protein modification on this reaction (Smith et al., 1981). On the one hand, changes in the electrostatics of the lipid–protein interaction are expected to mirror those in the electrostatic interaction between cytochrome *c* and cytochrome oxidase (albeit with oppositely charged groups). On the other hand, the association of the negatively charged cardiolipin with cytochrome oxidase is expected to enhance the electrostatic interaction with cytochrome *c*, and this potentiation of cytochrome binding will be modulated by similar effects to those influencing the lipid–protein interaction. Increasing ionic strength is indeed found to affect the catalytic activity of cytochrome oxidase, in a way that can be interpreted in terms of a decrease in the rate of the primary step involving association of cytochrome *c* (Brzezinski & Malmström, 1986). The enzyme activity also changes between pH 6.2 and pH 8.6, possibly due to histidine titration (Sinjorgo et al., 1986), and this may to a certain extent correlate with the results of Figure 5. Covalent modification of cytochrome *c* has also been found to affect the rate of enzymatic reaction with cytochrome oxidase (Smith et al., 1981), in a manner somewhat analogous to the effects that covalent modification of cytochrome oxidase has on the lipid–protein interaction.

Finally, it should be noted that the preferential association of the different cardiolipin analogues may have some significance for enzymatic function, since it has been demonstrated that lipids which tend to form nonlamellar phases are especially effective in reconstitution of coupled activity for the Ca^{2+}-ATPase (Navarro et al., 1984). The different cardiolipin analogues studied here have been found to display a particularly rich nonlamellar phase behavior (Powell & Marsh, 1985), and it is possible that the enhanced concentration in the neighborhood of the protein may potentiate any possible activating effects.

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Low pH Induced Membrane Fusion of Lipid Vesicles Containing Proton-Sensitive Polymer†

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ABSTRACT: For the purpose of cytoplasmic delivery of aqueous content in liposomes through endosomes, we synthesized a pH-sensitive polymer, cetylacetyl(imidazol-4-ylmethyl)polyethylenimine (CAIPEI), which generates polycations at acidic pH. CAIPEI in its aqueous phase caused aggregation of sonicated vesicles composed of phosphatidylserine (PS) and phosphatidylcholine (PC) (molar ratio 1:4) when the pH of the solution was lowered. The polymer also induced membrane intermixing as measured by resonance energy transfer between vesicles containing *N*-(7-nitro-2,1,3-benz[*d*]oxadiazol-4-yl)phosphatidylethanolamine and those containing *N*-Rhodamine phosphatidylethanolamine at pH 4–5, while the addition of CAIPEI caused neither aggregation of PC vesicles nor the intermixing of liposomal membranes between PC and PC/PS vesicles at any pH. The CAIPEI-induced membrane intermixing was dependent on the polymer/vesicle ratio rather than on the polymer concentration. Then the polymer was incorporated into the bilayers of PC vesicles. These CAIPEI vesicles also caused membrane intermixing with liposomes containing PS under acidic conditions. The reconstituted CAIPEI did not reduce the trapping efficiency of vesicles or increase their permeability to glucose even at low pH. The vesicles caused the low pH induced aggregation and membrane intermixing with other negatively charged liposomes containing phosphatidic acid or phosphatidylglycerol. These results suggest that the protonation of the polymer at acidic pH endows the CAIPEI vesicles with the activity to fuse with negatively charged liposomes.

Many applications of liposomes as microcapsules for drug delivery and as tools for microinjection have been investigated. For the purpose of cytoplasmic delivery via the endosome of molecules encapsulated in liposomes, several pH-sensitive liposomes have been developed (Connor et al., 1984; Diacovo et al., 1986; Collins & Huang, 1986). This delivery system mimics the process that is believed to be used for delivery of nucleocapsids into the cell cytosol by certain enveloped viruses (White et al., 1983). These viruses bind to cell surface receptors and are then internalized by endocytosis. Following these events, viral envelopes fuse with endosomal membranes due to the acidic condition of endosomes, and the nucleocapsid is released into the cytosol. [In the case of nonenveloped viruses, nucleocapsid release might occur through the pH-sensitive damage to the endosomal membrane induced by these viruses (Seth et al., 1984; Blumenthal, 1986).] Furthermore, vesicular stomatitis virus (Yamada & Ohnishi, 1986; Eidelman et al., 1984), whose receptor was suggested to be the lipid bilayer itself (Yamada & Ohnishi, 1986), and influenza virus (Stegmann et al., 1985) were reported to fuse with negatively charged liposomes under acidic conditions. Other proteins such as the HN protein of Sendai virus (Chejanovsky et al., 1986) or clathrin (Blumenthal et al., 1983) are also reported to induce pH-dependent membrane fusion.

Here we report the effects of a synthetic amphiphathic polymer, cetylacetyl(imidazol-4-ylmethyl)polyethylenimine, on acidic phospholipid vesicles. This polymer is readily incorporated into liposomal membranes and becomes cationic by protonation at pH 4–6. The resulting polycation is expected to induce membrane fusion, since many polycations such as mellitin (Eytan & Almary, 1983), polymyxin B (Gad & Eytan, 1983), polylysine (Gad et al., 1982; Lampe & Nelsestuen, 1983), polyhistidine (Wang & Huang, 1984; Uster & Deamer, 1985), and other synthetic polycations (Oku et al., 1986) are known to fuse negatively charged liposomes. Evidence is presented to show that the polymer endows liposomes with fusing activity only at low pH.

Experimental Procedures

Materials. Egg yolk phosphatidylcholine (PC),[1] phosphatidic acid (PA) from egg yolk PC, phosphatidylglycerol (PG) from egg yolk PC, and phosphatidylethanolamine (PE) from hydrated egg yolk PC were gifts of the Research Institute of

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[1] Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; N-NBD-PE, *N*-(7-nitro-2,1,3-benz[*d*]oxadiazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine; PEI, polyethylenimine; CAIPEI, cetylacetyl(imidazol-4-ylmethyl)polyethylenimine.

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