*Biochimica et Biophysica Acta*, 941 (1988) 287–292
Elsevier



BBA 70562

**BBA Report**

# Spin label studies on the selectivity of lipid–protein interaction of cardiolipin analogues with the $Na^+/K^+$-ATPase

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(Received 11 February 1988)

Key words: Lipid–protein interaction; Lipid specificity; Cardiolipin; ATPase, $Na^+/K^+$-; Spin label; ESR

**The selectivity of lipid–protein interaction for various spin-labelled cardiolipin analogues in $Na^+/K^+$-ATPase membranes from *Squalus acanthias* has been investigated by ESR spectroscopy. Cardiolipin derivatives with different numbers of acyl chains, or in which the headgroup charge has been removed by methylation of the phosphate groups, all show a pronounced selectivity relative to phosphatidylcholine. Maximally three times more of the cardiolipin analogue is associated with the protein, than is phosphatidylcholine. The selectivity pattern in the absence of salt is in the order: cardiolipin ≈ monolysocardiolipin ≥ acylcardiolipin > dimethylcardiolipin ≫ phosphatidylcholine, where acylcardiolipin has the spin label chain attached to the centre-OH group of the headgroup. The degree of association of the negatively charged cardiolipins with the protein is reduced by salt, corresponding to the lower selectivity for dimethylcardiolipin. It is concluded that the selectivity of the $Na^+/K^+$-ATPase for cardiolipin is not solely of electrostatic origin, nor is it likely to originate in the larger number of fatty acid chains relative to diacyl phospholipids.**

## Introduction

The $Na^+/K^+$-ATPase is an integral active transport protein involved in the maintenance of ion concentration levels and osmotic regulation in the cell. The enzyme has been found to be activated optimally by various negatively charged phospholipids [1–6]. Spin label ESR experiments have also demonstrated that the protein displays a selectivity for certain negatively charged lipids [7–11], which correlates to some extent with the activity measurements [12]. Amongst these negatively charged lipids is the tetraacyl phospholipid cardiolipin (diphosphatidylglycerol) [8,10], which also is found to display a selectivity for several other membrane proteins, including cytochrome oxidase [10,13–15].

In the present study we have further investi-

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Abbreviations: 14-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-(14-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearoyl)-*sn*-glycero(3)phospho]-*sn*-glycerol; 14-lysoCLSL, 1-[1-acyl-2-lyso-*sn*-glycero-(3)phospho]-3-[1-acyl-2-(14-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearoyl)-*sn*-glycero(3)-phospho]-*sn*-glycerol; 14-acyl-CLSL, 1-(3-*sn*-phosphatidyl)-2-*O*-[14-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearoyl]-3-(3-*sn*-phosphatidyl)-*sn*-glycerol; 14-diMeCLSL, 1-[1,2-diacyl-*sn*-glycero(3)-*O*-methylphospho]-3-[1-acyl-2-(14-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearoyl)-*sn*-glycero(3)-*O*-methylphospho]-*sn*-glycerol; $Na^+/K^+$-ATPase, sodium and potassium activated adenosinetriphosphatase (EC 3.6.1.3); $C_{12}E_8$, octaethylene glycol dodecyl monoether; CDTA, *trans*-1-2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid; ESR, electron spin resonance.

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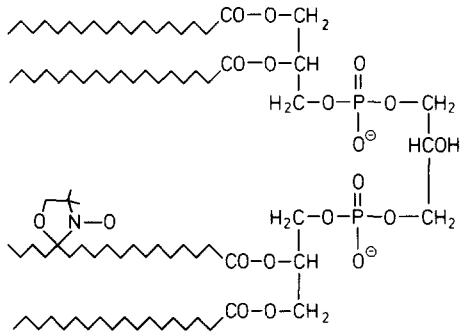
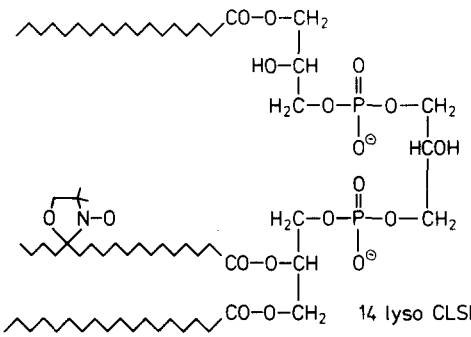
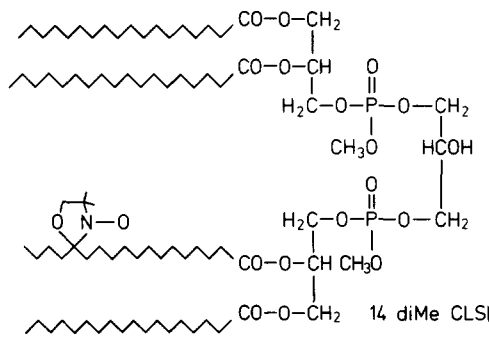


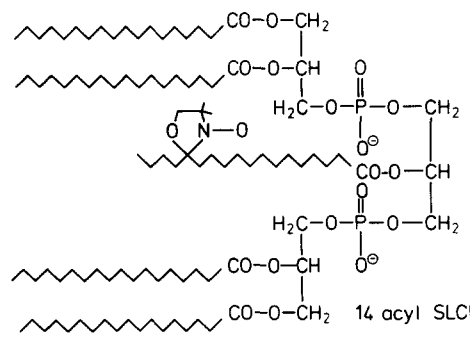
14 CLSL



14 lyso CLSL



14 diMe CLSL



14 acyl SLCL

gated the specificity of the $Na^+/K^+$-ATPase for cardiolipin by extending our previous spin label studies to analogues of cardiolipin bearing different numbers of fatty acid chains, or in which the polar group charge of the lipid is removed by methylation of the phosphate groups (cf. Refs. 13, 14). These cardiolipin derivatives are of additional interest since the unlabelled analogues have been shown to possess a rich polymorphic phase behaviour when dispersed in water and monovalent ion solutions [16].

## Materials and Methods

The spin-labelled derivatives of cardiolipin: 14-CLSL, 14-lysoCLSL, and 14-diMeCLSL, were labelled on the 14-C atom of the *sn*-2 chain (stearic acid), and the 14-acylCLSL derivative was labelled on the 14-C atom of stearic acid which was acylated to the centre hydroxyl moiety of the polar headgroup glycerol (see Fig. 1 for structures). The synthesis of the spin labels is described in Ref. 14.

$Na^+/K^+$-ATPase rich membranes from the rectal gland of *Squalus acanthias* were prepared as described in Ref. 18, but omitting the treatment with saponin. The $Na^+/K^+$-ATPase constituted typically 50–70% of the protein (determined as the content of $\alpha$- and $\beta$-subunits from SDS gel electrophoresis), and the specific activity ranged accordingly from 1100 to 1500 $\mu$mol ATP hydrolysed/mg protein per h. Since solubilization of these preparations in the detergent $C_{12}E_8$ yields an insoluble precipitate, presumably of peripheral proteins, and a supernatant which contains practically pure $Na^+/K^+$-ATPase [21], it can be assumed that the $Na^+/K^+$-ATPase is the only integral protein in the membrane. Extraction of the membrane lipids and the spin labelling of the $Na^+/K^+$-ATPase membranes and extracted lipids was performed as described in Refs. 8 and 9. The buffer used for suspending and pelleting the ESR samples was 10 mM Tris/1 mM EDTA (pH 7.4)

Fig. 1. Structures of cardiolipin spin label analogues. From top to bottom: cardiolipin, 14-CLSL; lysocardiolipin, 14-lysoCLSL; dimethyl cardiolipin, 14-diMeCLSL; acyl cardiolipin, 14-acyl-CLSL.

or 0.1 M NaCl/30 mM histidine/1 mM CDTA (pH 7.4).

ESR spectra were recorded on a Varian E-12 9 GHz spectrometer equipped with nitrogen gas flow temperature regulation. Further details of the ESR spectroscopy are given in Refs. 8 and 9. Spectral subtractions and data analysis were performed as described in Ref. 19.

## Results and Discussion

ESR spectra of the different spin-labelled cardiolipin analogues in $Na^+/K^+$-ATPase membranes, suspended both in 0.0 M NaCl and in 0.1 M NaCl, are given in Fig. 2. In each case the spectra consist of two components. One component corresponds to the fluid lipid environment characteristic of the spin-labels in the extracted membrane lipids (spectra not shown). The other component is indicated by the peaks in the outer wings of the spectra, and arises from the spin-labelled lipids interacting directly with the intramembranous surface of the integral membrane protein [15,17]. The upper spectrum of each pair is from membranes in 0.0 M NaCl and is seen to contain more of this second component than does the lower spectrum of each pair which is from membranes in 0.1 M NaCl. These differences correspond to the salt dependence found previously in the selectivity of other negatively charged lipids for the $Na^+/K^+$-ATPase [7,9]. Salt gives rise to ionic screening of the electrostatic interaction between the charged lipid and the protein, hence reducing the selectivity of the lipid–protein interaction [7,9,15]. Clearly there is a screenable electrostatic component in the selectivity of the charged cardiolipin analogues for the $Na^+/K^+$-ATPase *.

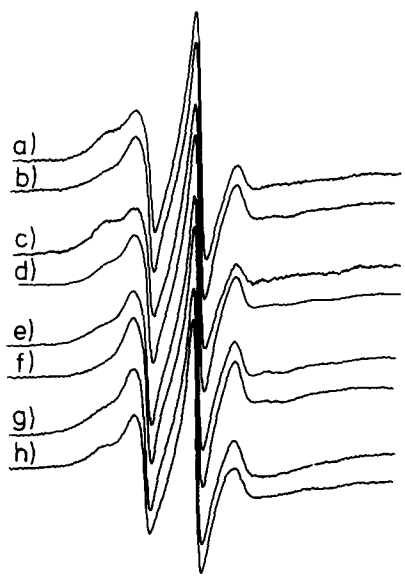



Fig. 2. ESR spectra of cardiolipin analogues, spin-labelled at the C-14 position, in $Na^+/K^+$-ATPase membranes from *Squalus acanthias*. The upper spectrum of each pair is in the absence of salt, and the lower is in 0.1 M NaCl. (a) cardiolipin, 14-CLSL, in 0.0 M NaCl; (b) 14-CLSL in 0.1 M NaCl; (c) lysocardiolipin, 14-lysoCLSL, in 0.0 M NaCl; (d) 14-lysoCLSL in 0.1 M NaCl; (e) acylcardiolipin, 14-acylCLSL, in 0.0 M NaCl; (f) 14-acylCLSL in 0.1 M NaCl; (g) dimethyl cardiolipin, 14-diMeCLSL, in 0.0 M NaCl; (h) 14-diMeCLSL in 0.1 M NaCl. Total scan width = 100 gauss; $T = 12°$ C.

The temperature dependence of the ESR spectra of the spin-labelled lysocardiolipin analogue in $Na^+/K^+$-ATPase membranes in the absence of salt is given in Fig. 3. This temperature dependence is typical for all analogues studied. It is seen that the fluid component, in the central regions of the spectrum, has a much steeper temperature dependence than that of the motionally restricted component, in the outer wings. The fluid component narrows considerably with increasing temperature, whereas the outer splitting of the motionally restricted component decreases only slightly. This illustrates the difference in the chain dynamics of the lipids in the two environments [22]. The chain rotational rates in the fluid bilayer regions of the membranes lie in the optimal range of sensitivity of conventional nitroxide ESR spectroscopy ($\tau_R \approx$ 1 ns), but those for the protein-interacting component are in the slow motional regime ($\tau_R \approx 10$ ns) [17]. Because of the steep temperature variation of the fluid component there is an optimal temperature range for spectral resolution of the two com-

* It will be noted that the samples in the presence and in the absence of salt also contain small amounts of buffer and chelator which differ between the two systems. However, detailed studies of the salt and pH dependence of the lipid selectivity for this system, both in different buffers and in the presence and absence of chelators, have revealed that the selectivity is determined principally by the ionic strength and pH and is not particularly sensitive to the different buffers or chelators used [9].

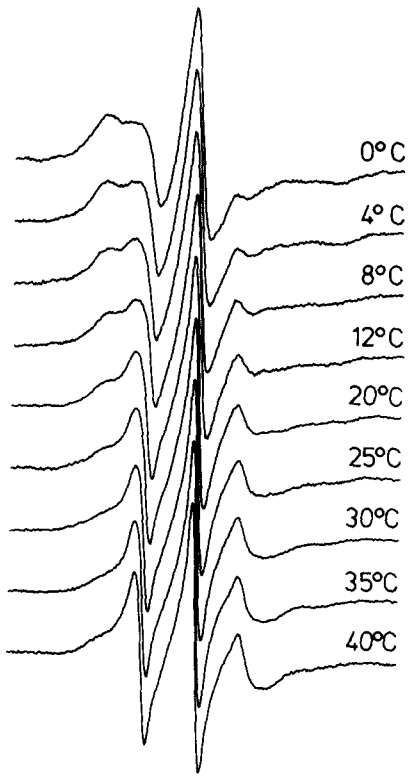



Fig. 3. Temperature dependence of the ESR spectra of the 14-lysoCLSL, lysocardiolipin spin label in $Na^+/K^+$-ATPase membranes in the absence of salt. Total scan width = 100 gauss.

ponents, and this has been chosen when quantitating the relative populations.

Spectral subtractions for the acylcardiolipin spin label derivative are illustrated in Fig. 4. Subtraction of the spectrum from the membrane in 0.1 M NaCl (Fig. 4b), which contains less of the motionally restricted component, from the spectrum of the membrane in 0.0 M NaCl (Fig. 4a) yields a motionally restricted difference spectrum as endpoint (Fig. 4c). Correspondingly, the reverse subtraction yields a fluid component difference spectrum for the endpoint (Fig. 4d). From the fractions ($1-k$ and $1-k'$) of the total double integrated intensity remaining in the two complementary difference spectra, it is possible to calculate the relative proportions of the motionally restricted and fluid components in the two original spectra used for the subtractions [7,17].

The fractions of the motionally restricted component, $f$, for the different labels at the two salt concentrations, that were obtained in the above way (which is defined as intersubtraction), are given in Table I. For comparison, the values of $f$ obtained by direct subtraction of the motionally restricted component, using a spectrum from dimyristoylphosphatidylcholine vesicles in the gel phase, are also given in Table I. As can be seen, the two different methods of subtraction, direct and intersubtraction, yield essentially consistent values for $f$. This gives an estimate of the inherent degree of precision in the subtraction endpoints. The reproduceability of the data can be assessed from comparison with previous experiments using the 14-CLSL label, in which $f$ was found to be 0.52 in 0.1 M NaCl [8], compared with a value of

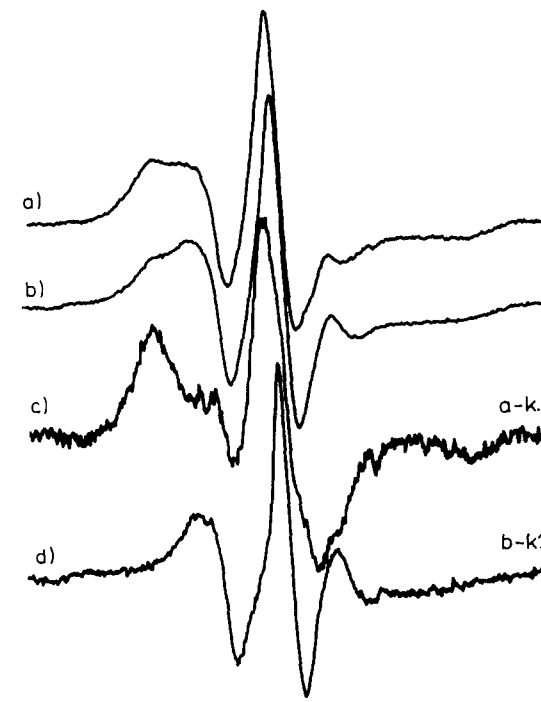



Fig. 4. Spectral subtraction for the 14-acylCLSL, acyl cardiolipin spin label in $Na^+/K^+$-ATPase membranes in 0.0 M and in 0.1 M NaCl, with 30 mM histidine, 1 mM CDTA, pH 7.4. (a) Spectrum from membranes in 0.0 M NaCl at 0 °C; (b) spectrum from membranes in 0.1 M NaCl at 0 °C; (c) motionally restricted component endpoint difference spectrum obtained by subtracting a fraction ($k$) of spectrum (b) from spectrum (a); (d) fluid component difference spectrum obtained by subtracting a fraction ($k'$) of spectrum (a) from spectrum (b). Total scan width = 100 gauss.

TABLE I

FRACTION OF MOTIONALLY RESTRICTED LIPID FOR SPIN-LABELLED CARDIOLIPIN ANALOGUES IN $Na^+/K^+$-ATPase MEMBRANES AT 0 °C, IN THE ABSENCE AND PRESENCE OF 0.1 M NaCl, pH 7.4.

| Spin label | 0.0 M NaCl | 0.1 M NaCl | |
|---|---|---|---|
| 14-CLSL | 0.62 [a] | 0.44 [a] | 0.49 [b] |
| 14-lysoCLSL | 0.67 [a] | 0.43 [a] | 0.49 [b] |
| 14-acylCLSL | 0.58 [a] | 0.44 [a] | 0.43 [b] |
| 14-diMeCLSL | – | – | 0.43 [b] |

[a] Obtained from intersubtractions of spectra for samples at 0 and 0.1 M NaCl.

[b] Obtained by subtracting a motionally restricted spectrum from the spectrum of samples in 0.1 M NaCl.

0.49 given in Table I for the same system and employing the same subtraction method.

Unfortunately, it was not found possible to obtain quantitatively reliable difference spectra for the 14-diMeCLSL label, because of residual spin-spin broadening of the membrane spectra in 0.0 M NaCl (cf. Fig. 2). This is presumably due to less efficient incorporation of 14-diMeCLSL into the membrane, because of the apolar nature of this label. The spin-spin broadened component in Fig. 2g could be removed by spectral subtraction, using a spectrum from pure spin label vesicles. However, an exact quantitative endpoint could not be obtained because the extreme width of the spin label vesicle spectrum causes rather large errors in the double integrated intensity of the difference spectrum for negligible differences in the lineheight. Thus comparisons of spectral lineshapes (but not integrated intensity) could be made by overlaying the difference spectrum with the other original spectra. This revealed that the spectrum of 14-diMeCLSL in the absence of salt in Fig. 2g contains a comparable proportion of motionally restricted component to the spectrum of the same label in 0.1 M NaCl in Fig. 2h, and this is considerably less than that for the 14-acylCLSL label in the absence of salt in Fig. 2e. The visual impression of the relative amounts of fluid and motionally restricted components obtained from Fig. 2, is therefore borne out by this comparison.

The values for the fraction of motionally restricted lipid in Table I reveal rather similar degrees of selectivity and response to salt for all three negatively-charged cardiolipin analogues. For comparison, the fraction of motionally restricted phosphatidylcholine spin label in similar membrane preparations was found to be $f \approx 0.22$ [8,10]. Clearly, all three cardiolipin analogues in Table I display a marked specificity relative to phosphatidylcholine. Although it was not possible to quantitate the spectra for 14-diMeCLSL accurately, it is clear from Fig. 2 and the data given immediately above that in 0.0 M NaCl this label exhibits a lower selectivity than the other cardiolipin analogues. However, a comparison with the spectra of the phosphatidylcholine spin label [8] reveals that the uncharged dimethylcardiolipin, nonetheless, has a pronounced specificity relative to zwitterionic phosphatidylcholine. For the direct subtraction in 0.1 M NaCl, the 14-diMeCLSL also reveals a somewhat smaller degree of association than the 14-CLSL and 14-lysoCLSL labels.

The result that varying the number of lipid acyl chains from 3 to 5 has little effect on the association of cardiolipin with the $Na^+/K^+$-ATPase shows that the greater number of chains cannot be per se the origin of the cardiolipin specificity *. In thermodynamic terms, this also means that the chain contributions to the energetics of the lipid–protein interaction are essentially the same as those for the lipid–lipid interactions in the fluid bilayer regions of the membrane. That is to say, the hydrophobic effect is of comparable strength in the two lipid environments [12–14]. It will also be noted that there is no cooperative effect of the number of chains on the lipid–protein interaction, contrary to the suggestion in Ref. 20. The rigid lattice model applied in the latter work is evidently inappropriate for the lipid/protein interface.

* It could be argued from the present data that a minimum of three chains is necesssary to obtain the observed selectivity for cardiolipin, but that addition of two further chains has little effect. This alternative seems less likely, partly also because stearic acid with only one chain shows a pronounced specificity in the same system [8,9]. In addition, rhodopsin which shows no specificity for a range of different phospholipid headgroups also shows no specificity resulting from the four chains of the cardiolipin molecule [10].

The reduction in the degree of association of the cardiolipin analogues, either by screening with salt or by removing the lipid charge on methylation, indicates that there is an appreciable electrostatic contribution to the selectivity of the $Na^+/K^+$-ATPase for cardiolipin. However, the fact that the dimethyl derivative still shows a specificity relative to phosphatidylcholine demonstrates a very significant nonelectrostatic contribution to the selectivity. This component presumably depends on the detailed headgroup structure and possibly also hydration. Some form of complementarity must exist between the lipid headgroup and protein structure, since for certain other integral membrane proteins, notably rhodopsin [10], cardiolipin does not display a selectivity.

We would like to thank Frau S. Schreiner for her skillful technical assistance. M.E. acknowledges financial support from the Danish Medical Research Council. G.L.P. was a Fulbright Visiting Professor, and acknowledges subsequent support from the National Science Foundation (U.S.A.-F.R.G. cooperative programme).

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