

## Lipid-Protein Interactions in ADP-ATP Carrier/Egg Phosphatidylcholine Recombinants Studied by Spin-Label ESR Spectroscopy

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**ABSTRACT:** The stoichiometry and specificity of lipid-protein interaction, as well as the lipid exchange rates at the protein interface, have been determined from the electron spin resonance spectra of spin-labeled lipids in reconstituted complexes of the mitochondrial ADP-ATP carrier with egg phosphatidylcholine. With the exception of cardiolipin and phosphatidic acid, the lipids studied are found to compete for approximately 50 sites at the intramembranous surface of the protein dimer. This number of first-shell lipid sites is unusually large for a protein of this size. The specificity for the protein is in the order stearic acid  $\approx$  phosphatidic acid  $\approx$  cardiolipin  $>$  phosphatidylserine  $>$  phosphatidylglycerol  $\approx$  phosphatidylcholine, with the maximum association constant relative to phosphatidylcholine being approximately 4. The selectivity for anionic lipids was partially screened with increasing ionic strength, but to a lesser extent for cardiolipin and phosphatidic acid than for stearic acid. Only in the case of phosphatidylserine was the selectivity reduced at high ionic strength to a level close to that for phosphatidylcholine. The off rates for lipid exchange at the protein surface were independent of lipid/protein ratio and correlated in a reciprocal fashion with the different lipid selectivities, varying from  $5 \times 10^6 \text{ s}^{-1}$  for stearic acid at low ionic strength to  $2 \times 10^7 \text{ s}^{-1}$  for phosphatidylcholine and phosphatidylglycerol. The off rates for cardiolipin were unusually low in comparison with the observed selectivity, and indicated the existence of a special population of sites (ca. 30% of the total) for cardiolipin, at which the exchange rate was very low. These results are discussed with reference to earlier observations on this hydrophobic membrane protein.

The ADP-ATP carrier is the most abundant integral protein of the inner mitochondrial membrane, in which it is responsible for the coupled transport of ADP and ATP in a counterexchange fashion. The molecular weight of the protein is 32 821; it consists of a single polypeptide chain and is thought to be present in the membrane in a dimeric form (Riccio et al., 1975a; Hackenberg & Klingenberg, 1980; Horváth et al., 1989). The amino acid composition of the ADP-ATP carrier is characterized by a high proportion of both hydrophobic and basic residues which are arranged in the primary sequence in a manner that can be matched to the amphiphilic profile of a phospholipid bilayer (Bogner et al., 1986). It thus may be expected that the carrier will interact strongly with the anionic lipids of the inner mitochondrial membrane in which it is incorporated. Of particular interest is the interaction with cardiolipin (diphosphatidylglycerol), a phospholipid which is unique to mitochondria in mammalian systems.

Previous studies have revealed a tight association of approximately six molecules of endogenous cardiolipin with the solubilized nucleotide carrier (Beyer & Klingenberg, 1985). Recently, it could be demonstrated by adding radioactive cardiolipin that the added and endogenous cardiolipin molecules do not exchange readily (M. Schlame and K. Beyer, unpublished results). Binding of spin-labeled phospholipids to the detergent-solubilized carrier has been investigated by using electron spin resonance (ESR)<sup>1</sup> spectroscopy (Drees & Beyer, 1988). It was found that two molecules of spin-labeled cardiolipin were bound with high affinity to each carrier dimer. A similar high affinity was found for phosphatidic acid, but not for zwitterionic phosphatidylcholine or negatively charged

phosphatidylglycerol. When considering the above-mentioned result, it must be assumed that the spin-labeled lipid molecules bind in addition to the tightly bound endogenous cardiolipin.

Experiments on the reconstituted carrier have also demonstrated a stimulation of activity by negatively charged phospholipids, particularly cardiolipin (Krämer & Klingenberg, 1980; Brandolin et al., 1980). However, an interpretation of the transport studies is complicated, since the effect of both surface potential and direct lipid-protein interaction must be considered (Krämer, 1983).

In the present work, we have investigated the lipid-protein interactions with the purified ADP-ATP carrier in complexes with egg phosphatidylcholine, using ESR spectroscopy of spin-labeled phospholipid species. Unlike the experiments with the detergent-solubilized system, this allows the investigation of all lipid association sites, both low affinity and high affinity, in the membrane-bound form of the carrier. Both the stoichiometry of the lipid sites at the protein interface, the selectivity of interaction of different lipid species with the protein, and the exchange rate of the lipids at the protein-associated sites have been determined. A significant result of these studies is that a proportion of the spin-labeled cardiolipin appears to be associated at sites on the ADP-ATP carrier for which its exchange rate is very much slower than the normal diffusive lipid motions in membranes.

<sup>1</sup> Abbreviations: 14-PCSL, -PGSL, -PSSL, and -PASL, 1-acyl-2-[13-(4,4-dimethyl-3-oxy-2-butyl-2-oxazolidinyl)tridecanoyl]-sn-glycero-3-phosphocholine, -phosphoglycerol, -phosphoserine, and phosphoric acid, respectively; 14-SASL, 14-(4,4-dimethyloxazolidine-N-oxyl)stearic acid; 14-CLSL, 1-(3-sn-phosphatidyl)-3-[1-acyl-2-O-[13-(4,4-dimethyl-3-oxy-2-butyl-2-oxazolidinyl)tridecanoyl]-sn-glycero-3-phospho]-sn-glycerol; egg PC, phosphatidylcholine from egg yolk; MOPS, 4-morpholinepropanesulfonic acid; ESR, electron spin resonance; ST-ESR, saturation-transfer ESR; L/P, lipid/protein mole ratio.

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## MATERIALS AND METHODS

**Materials.** Egg yolk phosphatidylcholine (egg PC) was obtained from Lipid Products (South Nutfield, U.K.). Spin-labeled stearic acid (14-SASL) was synthesized according to the method of Hubbell and McConnell (1971). The spin-labeled phospholipids 14-PCSL, -PASL, -PSSL, and -PGSL were synthesized from 14-SASL and egg lyso-PC as described in Marsh and Watts (1982). Spin-labeled cardiolipin (14-CLSL) was prepared by reacylation of monolysocardiolipin with 14-SASL (Cable & Powell, 1980). The purity of the spin-labeled phospholipids was checked by thin-layer chromatography.

**Isolation and Reconstitution of ADP-ATP Carrier.** The ADP-ATP carrier protein was isolated from beef heart mitochondria as described by Riccio et al. (1975b). Protein incorporation into egg phosphatidylcholine membranes was performed essentially as described previously (Beyer & Munding, 1986). Approximately 15 mg of the carrier protein in 18 mL of 200 mM NaCl, 10 mM MOPS, and 0.6% Triton X100 was added to a dry phospholipid film of 60 mg of egg phosphatidylcholine. Excess Triton was removed by treatment with 7 g of Amberlite XAD beads. The membranes were spun down, and protein addition was repeated once or twice in order to obtain lower lipid/protein ratios. Removal of excess phospholipid liposomes was achieved by one of two different methods. Preparations containing a high proportion of protein to phospholipid were centrifuged in D<sub>2</sub>O buffer. The thick supernatant containing almost protein-free phospholipid was discarded. With lower protein/lipid ratios, a homogeneous liposome preparation was obtained by using a sucrose density gradient (Beyer & Klingenberg, 1983). Protein, phospholipid, and Triton X100 were determined as described previously (Beyer & Klingenberg, 1985).

**ESR Spectroscopy.** ESR measurements were carried out at 9 GHz using a Varian E-12 ESR spectrometer with nitrogen gas flow temperature regulation. Samples were contained in 1-mm i.d. glass capillaries which were immersed in silicone oil to improve thermal stability (typically  $\pm 0.1$  °C). Spectral subtractions were carried out as described previously [for a review, see Marsh (1982)].

**Spectral Simulations.** Spectral simulations were based on a two-site chemical exchange model as described in Marsh and Horváth (1989). The variable parameters in the simulation are  $\tau_b^{-1}$  and  $\tau_f^{-1}$ , which are the probabilities per unit time of transfer from a protein-associated site (b) to any of the bulk lipid sites (f) and vice versa. At exchange equilibrium, these are related by (Horváth et al., 1988a)

$$f\tau_b^{-1} = (1-f)\tau_f^{-1} \quad (1)$$

where  $f$  is the fraction of motionally restricted lipid component associated with interfacial sites on the protein. It is assumed that there is a random reorientation of the lipid acyl chains on collision with the invaginated protein interface and hence the angular orientation of the spin-label is uncorrelated before and after the exchange; this is essentially model II of Davoust and Devaux (1982). Details of the fitting procedure are given in Horváth et al. (1988b).

## RESULTS

**Stoichiometry of ADP-ATP Carrier/Lipid Interaction.** All of the ESR spectra of the spin-labeled lipids in the lipid-protein recombinants consisted of two components, when recorded at a temperature above the gel-fluid-phase transition of egg PC. Resolution of the components by intersubtraction between spectra containing different proportions of the two components is illustrated in Figure 1. One component (Figure 1c) cor-

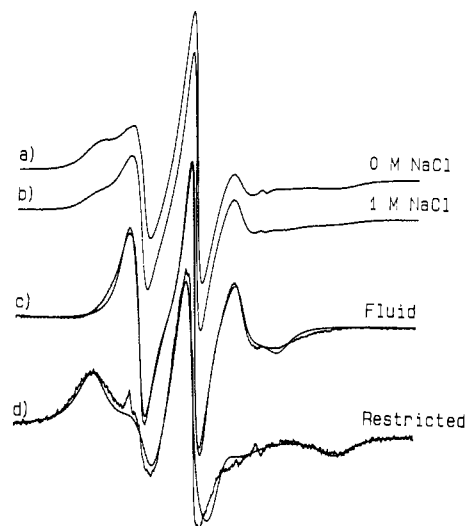


FIGURE 1: Intersubtractions between the ESR spectra of 14-SASL stearic acid spin-label in recombinants of the ADP-ATP carrier with egg PC at a lipid/protein ratio of 106/1 mol/mol, in 0.0 M NaCl and in 1.0 M NaCl.  $T = 10$  °C. (a) Experimental spectrum in 0.0 M NaCl; (b) experimental spectrum in 1.0 M NaCl; (c) fluid component end point obtained by subtracting (a) from (b) (solid line) and simulated single-component spectrum (dashed line); (d) motionally restricted component end point obtained by subtracting (b) from (a) (solid line) and simulated single-component spectrum (dashed line). Total scan width = 100 G.

responds to the bulk fluid lipid regions in the recombinant and the other (Figure 1d) to the lipids interacting directly with the intramembranous hydrophobic surface of the protein [cf. Marsh (1985)]. Similar values for the relative proportions of the two components were also obtained by subtracting a matching single-component spectrum of fluid egg PC from the individual two-component spectra (data not shown).

The ESR spectra of the 14-PSSL spin probe in ADP-ATP carrier/egg phosphatidylcholine (PC) recombinants at different L/P ratios are shown in Figure 2. An increasing proportion of the motionally restricted spectral component with increasing protein content in the recombinants is clearly seen. The incorporation of the ADP-ATP carrier into egg PC vesicles and the homogeneity of the lipid-protein complexes were confirmed by continuous sucrose density gradient centrifugation. All protein-lipid complexes sedimented to an extent determined by their L/P ratios, and samples in which traces of pure lipid liposomes or contamination by protein aggregates could be detected were excluded from subsequent spectral analyses.

The results of such lipid/protein ratio titration experiments with 14-PCSL, 14-PGSL, 14-PSSL, and 14-SASL spin-labeled lipids are given in Figure 3. The titrations are fitted to the approximate equation for protein-lipid association (Brotherus et al., 1981; Knowles et al., 1979):

$$n_f^*/n_b^* = (1/K_r)(n_t/N_1 - 1) \quad (2)$$

where  $n_f^*/n_b^* = (1-f)/f$  is the ratio of the double-integrated intensities of the fluid and motionally restricted components,  $n_t$  is the total lipid to protein ratio of the complex,  $N_1$  is the average number of lipid solvation sites at the protein interface, and  $K_r$  is the average association constant of the phospholipid spin probe with the protein, relative to that of the host lipid (egg PC). For 14-PCSL and 14-PGSL, the intercept on the ordinate gives  $K_r(\text{PC}) = 1.0$ , indicating no selectivity relative to the background egg PC. The number of lipid solvation sites is obtained from the intercept on the abscissa giving  $N_1 = 50$  for the ADP-ATP carrier dimer. 14-PSSL and 14-SASL have clear selectivities over phosphatidylcholine with average relative

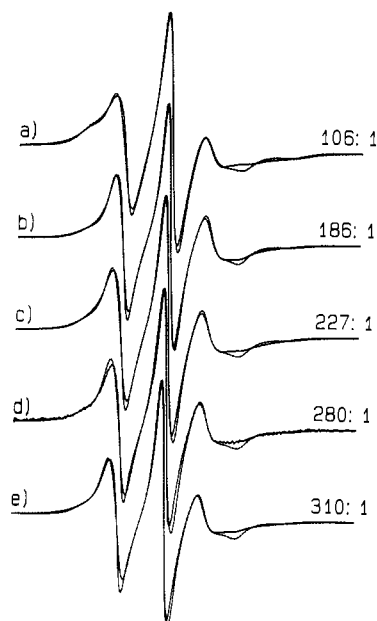


FIGURE 2: ESR spectra of the 14-PSSL phosphatidylserine spin-label in ADP-ATP carrier recombinants with egg phosphatidylcholine at various lipid/protein mole ratios.  $T = 10^\circ\text{C}$ . (a) 106/1 mol/mol; (b) 186/1 mol/mol; (c) 227/1 mol/mol; (d) 280/1 mol/mol; (e) 310/1 mol/mol. Solid lines are the experimental spectra, and dashed lines are simulations from the two-component exchange model. Total scan width = 100 G.

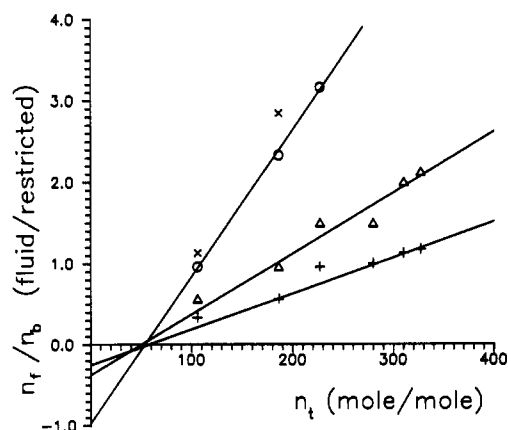


FIGURE 3: Lipid-protein titration of ADP-ATP carrier in egg PC recombinants from ESR difference spectra of the 14-PCSL phosphatidylcholine (O), 14-PGSL phosphatidylglycerol (X), 14-PSSL phosphatidylserine (Δ), and 14-SASL stearic acid (+) spin-labels at  $10^\circ\text{C}$ .  $n_r/n_b$  is the ratio of the double-integrated intensity of the fluid and motionally restricted components in the ESR spectra of the recombinants, and  $n_t$  is the total lipid/protein ratio. Solid lines are linear regressions, according to eq 2, except that the two experimental points clearly above the line for 14-SASL were omitted from the regression.

association constants of  $K_r(\text{PS}) = 2.4$  and  $K_r(\text{SA}) = 4.1$ , respectively. The corresponding numbers of association sites are not significantly different from that of 14-PCSL ( $N_1 = 51 \pm 8$ ).

In addition, 14-CLSL and 14-PASL were also studied. From the spectra given later in Figure 4, it is clear that both of these lipids display a selectivity for the protein that is either comparable to or greater than that of 14-SASL. However, the dependence on lipid/protein ratio does not conform to the simple association model given in eq 2, in that the values of  $n_r/n_b$  tend to a constant limiting value at high lipid/protein ratio (data not shown). A possible reason for this could be the presence of a limited number of association sites of much higher selectivity for these two lipids. This type of saturation

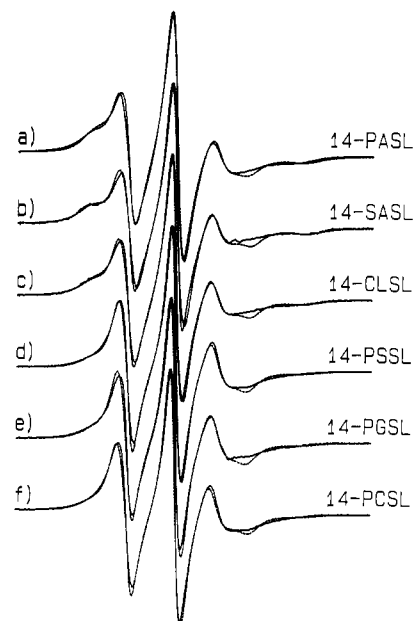


FIGURE 4: ESR spectra of the different headgroup spin-labels in ADP-ATP carrier recombinants with egg phosphatidylcholine of lipid/protein ratio 186/1 mol/mol, recorded at  $10^\circ\text{C}$ . (a) 14-PASL phosphatidic acid spin-label; (b) 14-SASL stearic acid spin-label; (c) 14-CLSL cardiolipin spin-label; (d) 14-PSSL phosphatidylserine spin-label; (e) 14-PGSL phosphatidylglycerol spin-label; (f) 14-PCSL phosphatidylcholine spin-label. Solid lines are the experimental spectra, and dashed lines are simulations from the two-component exchange model. Total scan width = 100 G.

Table I: On- ( $\tau_r^{-1}$ ) and Off-Rate Constants ( $\tau_b^{-1}$ ) for Lipid Exchange of Spin-Labeled Phospholipids and Stearic Acid at the Interface of the Mitochondrial ADP-ATP Carrier in Egg PC Complexes of Various Lipid to Protein Ratios in Low-Salt Buffer ( $<0.01\text{ M NaCl}$ ), pH 7.4,  $T = 10^\circ\text{C}$ <sup>a</sup>

spin-label	L/P (mol/mol)	$f$	$\tau_r^{-1} (\text{s}^{-1})$	$\tau_b^{-1} (\text{s}^{-1})$
14-PCSL	106	0.51	$1.4 \times 10^7$	$1.7 \times 10^7$
	186	0.30	$1.4 \times 10^7$	$1.9 \times 10^7$
14-PGSL	186	0.26	$1.4 \times 10^7$	$1.8 \times 10^7$
	186	0.64	$1.3 \times 10^7$	$1.0 \times 10^7$
14-PSSL	106	0.51	$9.9 \times 10^6$	$9.5 \times 10^6$
	227	0.40	$7.4 \times 10^6$	$1.0 \times 10^7$
	280	0.40	$5.6 \times 10^6$	$8.4 \times 10^6$
	310	0.33	$5.0 \times 10^6$	$9.1 \times 10^6$
	327	0.32	$4.5 \times 10^6$	$9.5 \times 10^6$
	327	0.64	$8.3 \times 10^6$	$4.6 \times 10^6$
14-SASL	186	0.65	$1.3 \times 10^7$	$6.8 \times 10^6$
14-CLSL	186	0.62	$9.0 \times 10^6$	$5.5 \times 10^6$

<sup>a</sup> The fraction,  $f$ , of motionally restricted spin-label is obtained from spectral subtraction and the exchange rates from spectral simulation.

behavior observed in the lipid-protein titration for 14-CLSL and 14-PASL has been simulated previously for just such a high-affinity model by Brotherus et al. (1981). Data on the exchange rates for 14-CLSL also give further support to this interpretation (see Discussion).

**Lipid Specificity and Salt Titration.** The lipid specificity of the mitochondrial ADP-ATP carrier was studied by comparing the spectra of the different spin-labels in a complex of fixed lipid/protein ratio ( $L/P = 186\text{ mol/mol}$ ). The ESR spectra of the various 14-C-labeled lipids in the ADP-ATP carrier/egg PC recombinant are shown in Figure 4. The results from spectral subtractions and spectral simulations (see later) are summarized in Table I. Since the number of solvation sites  $N_1$  remains the same for these lipids (cf. Figure 3), the relative association constant can be determined directly from the ratios of the fluid/motionally restricted components:

$$(n_r/n_b)_{\text{PC}}/(n_r/n_b)_{\text{L}} = K_r(\text{L})/K_r(\text{PC}) \quad (3)$$

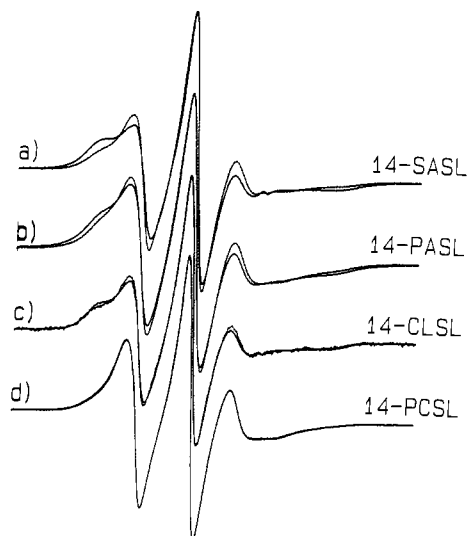


FIGURE 5: ESR spectra of the various headgroup spin-labels in ADP-ATP carrier recombinants with egg PC at a lipid/protein ratio of 106/1 mol/mol, in 0.0 M NaCl (solid lines) and in 1.0 M NaCl (dashed lines). (a) 14-SASL stearic acid spin-label; (b) 14-PASL phosphatidic acid spin-label; (c) 14-CLSL cardiolipin spin-label; (d) 14-PCSL phosphatidylcholine spin-label. Spectra recorded at  $T = 10^\circ\text{C}$ ; total scan width = 100 G.

Values of  $K_r(\text{CL}) = 3.8$ ,  $K_r(\text{PA}) = 4.3$ ,  $K_r(\text{SA}) = 4.1$ ,  $K_r(\text{PS}) = 2.4$ , and  $K_r(\text{PG}) = 0.8$  were obtained for 14-CLSL, 14-PASL, 14-SASL, 14-PSSL, and 14-PGSL, respectively, assuming that  $K_r(\text{PC}) = 1$ , as found above. The corresponding differential free energy of association,  $\Delta G^\circ(\text{L}) - \Delta G^\circ(\text{PC}) = -RT \ln [K_r(\text{L})/K_r(\text{PC})]$ , was  $-2.1$  and  $-3.3 \text{ kJ mol}^{-1}$  for 14-PSSL and 14-SASL, respectively, in agreement with the values obtained from the lipid-protein titration.

The salt dependence of the protein-lipid association of four different spin-labeled phospholipids in ADP-ATP carrier/egg PC complexes suspended in 10 mM MOPS buffer, pH 7.4, is illustrated in Figure 5. When the NaCl concentration was increased from  $<10 \text{ mM}$  to  $1.0 \text{ M}$  by resuspending the complexes in buffers of increasing NaCl concentrations, the relative intensity of the motionally restricted component decreased for 14-SASL, 14-CLSL, and 14-PASL to different extents, whereas no change was observed in the case of the zwitterionic 14-PCSL control. At the end of the salt titration experiments, the complexes were resuspended in the NaCl-free buffer, and essentially identical spectra were obtained with the initial spectra. The results of spectral subtractions are summarized by the values for the fraction,  $f$ , of motionally restricted spin-label component as a function of salt concentration that are given later in Table II. Somewhat similar results have also been obtained with the detergent-solubilized carrier protein (Drees, 1988).

**Spectral Simulations.** The fitting of the spectra for the fluid and motionally restricted single components is illustrated in Figure 1 [cf. Horváth et al. (1988)]. The line widths for Figure 1c were determined from pure lipid spectra (i.e., in the absence of exchange), recorded at the same temperature ( $10^\circ\text{C}$ ) as those of the lipid-protein complexes, but the effective order parameter was adjusted to match the increase in spectral anisotropy induced by the protein. A rigorous treatment of the discrepancy between experimental and simulated components, which is particularly obvious in the high-field part of the spectrum, would require a more general (slow motion) line shape formulation. For this reason, all exchange rates were determined by concentrating on the central and low-field lines in the fluid component, which display best sensitivity to exchange and good agreement between the experimental and

Table II: On- ( $\tau_r^{-1}$ ) and Off-Rate Constants ( $\tau_b^{-1}$ ) for Lipid Exchange of Spin-Labeled Phosphatidylserine, Stearic Acid, and Cardiolipin at the Interface of Mitochondrial ADP-ATP Carrier/Egg PC Complexes (L/P = 106 mol/mol) as a Function of Salt Concentration at pH 7.4,  $T = 10^\circ\text{C}$ <sup>a</sup>

spin-label	[NaCl] (M)	$f$	$\tau_r^{-1} (\text{s}^{-1})$	$\tau_b^{-1} (\text{s}^{-1})$
14-PSSL	0.01	0.64	$1.3 \times 10^7$	$1.0 \times 10^7$
	0.05	0.62	$1.5 \times 10^7$	$1.3 \times 10^7$
	0.1	0.61	$1.6 \times 10^7$	$1.5 \times 10^7$
	0.5	0.59	$1.6 \times 10^7$	$1.5 \times 10^7$
	1.0	0.56	$1.6 \times 10^7$	$1.5 \times 10^7$
14-SASL	0.01	0.75	$1.2 \times 10^7$	$5.2 \times 10^6$
	0.05	0.73	$1.5 \times 10^7$	$6.9 \times 10^6$
	0.1	0.70	$1.6 \times 10^7$	$8.0 \times 10^6$
	0.5	0.65	$1.6 \times 10^7$	$1.0 \times 10^7$
	1.0	0.62	$1.6 \times 10^7$	$1.2 \times 10^7$
14-CLSL	0.01	0.71	$9.3 \times 10^6$	$5.0 \times 10^6$
	0.05	0.70	$1.0 \times 10^7$	$5.9 \times 10^6$
	0.1	0.67	$1.1 \times 10^7$	$6.2 \times 10^6$
	0.5	0.65	$1.0 \times 10^7$	$6.4 \times 10^6$
	1.0	0.63	$1.1 \times 10^7$	$7.4 \times 10^6$

<sup>a</sup> The fraction,  $f$ , of motionally restricted spin-label is obtained from spectral subtraction and the exchange rates from spectral simulation.

simulated single-component spectra.

The simulated exchange-coupled two-component spectra which best fit the spectra of 14-PSSL from the L/P ratio titration are shown by dashed lines in Figure 2. Normalized mean square errors were less than 1.5% in every case. The on and off rates determined by these spectral simulations are summarized in Table I. It is found that the off rate is essentially independent of the L/P ratio. Further representative simulations for 14-PASL, 14-CLSL, 14-SASL, 14-PGSL, and 14-PCSL in ADP-ATP carrier/egg PC recombinants of L/P = 186 mol/mol are shown in Figure 4. The best-fitting parameters are given in Table I. Clearly, the lipid selectivity is reflected by the different off-rate constants for the different spin-labeled lipids, while the on rates remain approximately constant for a fixed lipid/protein ratio.

The on and off rates obtained by simulating the spectra from the salt dependence experiments are given in Table II (simulated spectra not shown). The on rates remain essentially constant with increasing salt concentration for all spin probes, although the fraction of motionally restricted component, and hence the lipid selectivity, varies considerably over this range of ionic strength. As the salt concentration is increased from  $<10 \text{ mM}$  to  $1 \text{ M}$  NaCl, the fraction of motionally restricted component is decreased, and, in parallel, the off rate is increased. It should be noted that the off rates for each acidic lipid displaying selectivity remain well below that obtained for 14-PCSL, even at the highest salt concentrations.

## DISCUSSION

**Stoichiometry of Lipid Association.** The results of the lipid-protein titrations indicate that there are approximately 50 lipid sites at the intramembranous surface of the protein dimer, for all the different lipids tested. This is an unusually high number for a protein of this size and correlates with the highly hydrophobic nature of the protein, suggesting that most of the protein mass is contained within the membrane. A similar conclusion was drawn previously on the basis of the unusually high detergent binding with the ADP-ATP carrier (Hackenberg & Klingenberg, 1980). About 150 molecules of Triton X100 bind to the isolated protein dimer.

The number of first-shell sites can be compared with the available structural data on the protein. From the hydrodynamic model of Hackenberg and Klingenberg (1980), the protein dimer can be estimated to have an elliptical cross section with semiaxes  $a = 23 \text{ \AA}$  and  $b = 20 \text{ \AA}$ , if it is assumed

that the dimer has the same asymmetry ( $a/b = 65/57$ ) as that induced in the protein-containing Triton micelle [cf. also Horváth et al. (1989)]. From this, it can be calculated that approximately 31 lipids can be accommodated around the intramembranous perimeter of the dimeric carrier. Alternatively, if it is assumed that all the dimer volume is contained solely within the membrane with a height of 45 Å, then  $a = 25$  Å and  $b = 22$  Å, and 34 lipids can be accommodated around the perimeter. These estimates of the cross-sectional dimensions of the dimer are consistent with rotational diffusion measurements on the ADP-ATP carrier (Horváth et al., 1989), but both yield a smaller estimate for the number of first-shell lipids than is deduced from the ESR measurements with lipid spin-labels. A possible explanation for this difference is that the surface is considerably more invaginated than assumed in the hydrodynamic model, and hence a further number of lipids can be additionally occluded at the protein surface. Such an explanation, in terms of the heterogeneity in lipid association sites, is consistent with the finding of an extra class of sites for cardiolipin (cf. below).

**Lipid Specificity.** The results of Table I indicate a marked selectivity for certain negatively charged phospholipids, in particular for cardiolipin. The results on selectivity can be readily correlated with the sequence and proposed structure of the protein (Klingenberg, 1985). Labeling experiments by Bogner et al. (1986) have located many of the lysine groups of this highly basic protein at the membrane surface, where they are able to interact directly with the phospholipid headgroups. This location of the charged protein residues is almost inevitable, since the protein is highly hydrophobic and most of its mass resides within the membrane.

**Lipid Exchange Rates.** The exchange rates at the lipid-protein interface display the expected dependence on lipid/protein ratio, selectivity, and ionic strength, hence substantiating the method used for the simulation. The off rates are independent of lipid/protein ratio, and the on rates at a fixed lipid/protein ratio are essentially independent of the selectivity of the different lipid headgroups and its screening by salt.

The off rates are modulated by the lipid specificity as demonstrated previously for myelin proteolipid protein (Horváth et al., 1988a) and M13 phage coat protein (Wolfs et al., 1989). At exchange equilibrium, eq 1 and 2 yield the following relation for the ratio of off- and on-rate constants:

$$\tau_b^{-1}/\tau_f^{-1} = (1/K_r)(n_i/N_i - 1) \quad (4)$$

From the results of the lipid selectivity series (Table I) and particularly from the salt dependence (Table II), it is deduced that the on-rate  $\tau_f^{-1}$  is essentially constant for a given lipid/protein ratio, because it is diffusion-controlled [cf. also Davoust et al. (1983)]. Hence, from eq 4, the ratio of off rates for different spin-labeled lipids, e.g., L and PC, at fixed lipid/protein ratio and fixed temperature can be written as

$$\tau_b^{-1}(\text{PC})/\tau_b^{-1}(\text{L}) = K_r(\text{L})/K_r(\text{PC}) \quad (5)$$

This inverse relation is shown in Figure 6 for the selectivity between the different phospholipids, and for the variation of their relative association constants with increasing NaCl concentration (data from Figures 4 and 5 and Tables I and II).

According to the correlation plot of  $\tau_b^{-1}$  vs  $1/K_r$ , all lipids conform to the inverse relation, except spin-labeled cardiolipin which displays significantly lower off rates for any given value of  $K_r$ . This indicates that part of the cardiolipin is associated at more highly specific sites for which the exchange rate is much slower than at the other boundary layer sites. Such an interpretation is fully consistent with the nonlinear lipid-protein

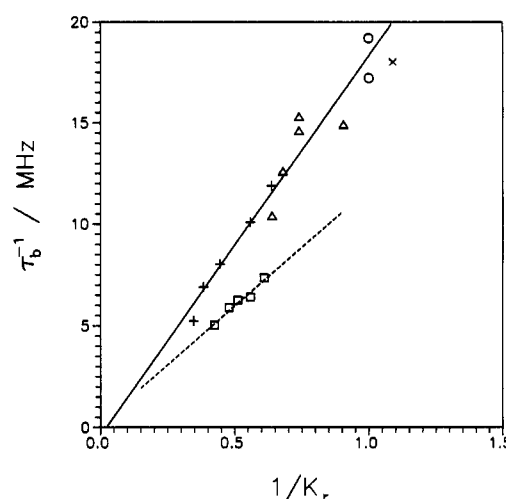


FIGURE 6: Correlation diagram between the relative association constant,  $K_r$ , and the off rate for exchange,  $\tau_b^{-1}$ , for the 14-SASL stearic acid (+), 14-PSSL phosphatidylserine ( $\Delta$ ), 14-PCSL phosphatidylcholine ( $\circ$ ), 14-PGSL phosphatidylglycerol ( $\times$ ), and 14-CLSL cardiolipin ( $\square$ ) spin-labels in ADP-ATP carrier/egg PC recombinants of lipid/protein ratio 106/1 mol/mol, at 10 °C. The different selectivities for a particular label correspond to different salt concentrations.

titration behavior of 14-CLSL described previously under Results. Assuming that the exchange rate at these specific sites is too low to produce any detectable broadening of the ESR spectrum ( $\tau_b^{-1} < 10^6 \text{ s}^{-1}$ ), it can be estimated that approximately 30% of the spin-labeled cardiolipin is associated at such sites. It should be noted that such slowly exchanging sites cannot be distinguished from the more rapidly exchanging sites by spectral subtractions, since both types of sites contribute to the motionally restricted component.

Previous  $^{31}\text{P}$  NMR studies have indicated that approximately six molecules of cardiolipin are tightly associated with the detergent-solubilized ADP-ATP carrier (Beyer & Klingenberg, 1985). The recent finding that exogenous radioactive cardiolipin does not readily exchange with this intrinsically bound cardiolipin (M. Schlame and K. Beyer, unpublished results) suggests that these sites are not accessible to the spin-labeled cardiolipin derivative. Thus, the high-affinity binding for cardiolipin (2 mol of cardiolipin per mole of protein dimer) found in ESR experiments analyzing the binding of various spin-labeled phospholipids with the solubilized ADP-ATP carrier (Drees & Beyer, 1988) must be attributed to additional association sites on the protein surface. This is corroborated by the observation that in the solubilized protein spin-labeled cardiolipin is easily displaced by nonlabeled cardiolipin but not by phosphatidylcholine (Drees, 1988). On the basis of these results, it can be concluded that a nonexchangeable pool of protein-bound cardiolipin also exists in the membranous environment.

**Salt Titration.** A semiquantitative description for the screening of the electrostatic component of the lipid-protein interaction for charged phospholipids at high ionic strength can be given by using Debye-Hückel theory. The ratio of the relative association constants,  $K_r/K_r^\circ$ , in the presence and absence of salt is approximated by (Esmann & Marsh, 1985; Horváth et al., 1988b)

$$\ln(K_r/K_r^\circ) = (35.7 \text{ nm}/r_D)[Z_L^2/(1 + a_{L^*}/r_D) + Z_P^2/(1 + a_P/r_D)] \quad (6)$$

where  $a_{L^*}$  and  $a_P$  are the molecular radii of lipids and proteins, respectively,  $r_D$  is the Debye radius for screening by monovalent ions ( $r_D^2 = 9.28 \times 10^{-20} \text{ m}^2 \text{ mol kg}^{-1}/I$ , where  $I$  is the

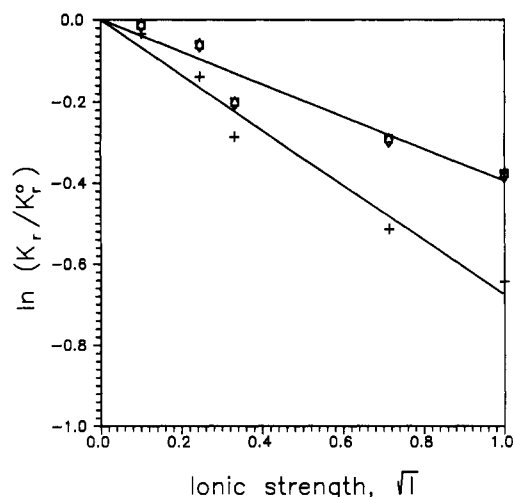


FIGURE 7: Salt dependence at pH 7.4 of the relative association constants,  $K_r$ , of the 14-SASL stearic acid (+), 14-PASL phosphatidic acid ( $\diamond$ ), and 14-CLSL cardiolipin ( $\square$ ) spin-labels in ADP-ATP carrier recombinants with egg PC of lipid/protein ratio 106/1 mol/mol. Experimental points are normalized to the value,  $K_r^0$ , in the absence of salt. Lines are theoretical curves deduced from Debye-Hückel theory, with  $Z_L = -1$ ,  $a_{SA} = 0.25$  nm, and  $a_{PA} = a_{CL} = 0.6$  nm, and shifted by 0.13 unit to correspond to the first experimental points.

ionic strength), and electroneutrality of the lipid-protein interface is assumed, i.e.,  $Z_p = -Z_L = 1$  or 2. The protein radius is taken to be  $a_p = 2.5$  nm, based on hydrodynamic measurements (Hackenberg & Klingenberg, 1980) and ST-ESR estimations (Horváth et al., 1989).

The logarithm of the ratio of the relative association constants is shown as a function of the square root of the ionic strength in Figure 7, together with the best-fitting theoretical curves. The salt dependence for stearic acid can be fitted satisfactorily with a value of  $a_L = 0.25$  nm, which is similar to that found previously with the myelin proteolipid protein (Horváth et al., 1988b) and to that used for cardiolipin interacting with cytochrome oxidase (Powell et al., 1987). For cardiolipin and phosphatidic acid, significantly higher values,  $a_L = 0.6$  nm, are required to obtain a good fit. These values are in agreement with that used for cardiolipin in Na,K-ATPase membranes (Esmann & Marsh, 1985) and are higher than those in myelin proteolipid protein/DMPC recombinants. Although the ionic strength dependence corresponds reasonably well to classical electrostatic screening, it should be emphasized that screening of the lipid charges does not entirely remove the selectivity of 14-CLSL, 14-PASL, and 14-SASL relative to 14-PCSL. This indicates that for the former three lipids there is also a nonelectrostatic contribution to the lipid-protein selectivity.

**Conclusions.** In summary, the ADP-ATP carrier possesses a large number of lipid association sites in relation to its size, indicating that most of the protein mass is integrated in the membrane. The protein exhibits a pronounced specificity for certain negatively charged lipids, and this specificity is in most cases only partly of direct electrostatic origin. More specific sites are available for cardiolipin at which the exchange rate is much slower. The latter points to a possible role for cardiolipin in the transport function of the ADP-ATP carrier.

**Registry No.** Stearic acid, 57-11-4.

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