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Chemistry and Physics of Lipids 141 (2006) 94–104
CPL
CHEMISTRY AND PHYSICS OF LIPIDS
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Review

Lipid–protein interactions with the Na,K-ATPase

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Received 18 November 2005; accepted 20 February 2006
Available online 20 March 2006

Abstract

Studies of lipid interactions with membranous Na,K-ATPase by using electron spin resonance spectroscopy in conjunction with spin-labelled lipids are reviewed. The lipid stoichiometry, selectivity and exchange dynamics at the lipid–protein interface can be determined, in addition to information on the configuration and rotational dynamics of the protein-associated lipid chains. These parameters, particularly the stoichiometry and selectivity, are related directly to the intramembranous structure of the Na,K-ATPase, and can be used to check the integrity of extensively trypsinised preparations.

Keywords: Lipid–protein interactions; Na,K-ATPase; Lipid selectivity; Spin labels; ESR

Contents

1. Introduction

Electron spin resonance (ESR) of spin-labelled lipids is uniquely useful in studying interactions with integral membrane proteins, and with the Na,K-ATPase (EC 3.6.3.9) in particular (Marsh and Watts, 1982; Marsh, 1985; Marsh and Horváth, 1989). The dynamic sensitivity of spin labelling is such that, even if the lipids at the intramembranous surface of the protein exchange at rates comparable to those in fluid lipid bilayers, they can be distinguished spectrally from the bulk fluid lipids (see Fig. 1). Thus, it is possi-

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doi:10.1016/j.chemphyslip.2006.02.018

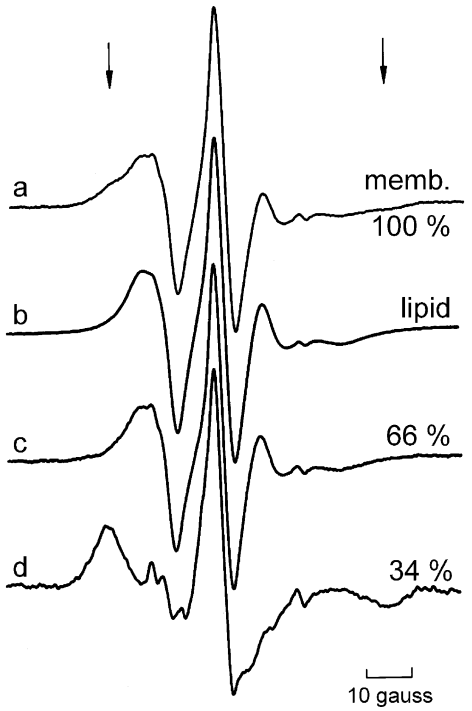

Fig. 1. Resolution of fluid and motionally restricted components in the ESR spectrum of C-14 spin-labelled stearic acid (14-SASL) in Na,K-ATPase membranes from *Squalus acanthias*. (a) 14-SASL in native membranes; (b) 14-SASL in bilayers of extracted lipids; (c) fluid difference spectrum obtained by subtracting a motionally restricted spectrum (dimyristoyl phosphatidylcholine vesicles in the gel phase) from (a); (d) motionally restricted difference spectrum obtained by subtracting (b) from (a). Relative intensities of the difference spectra are indicated on the figure. Scan width = 10 mT; $T = 0\,^{\circ}C$. Data from Marsh et al. (1982).

ble to quantitate both the stoichiometry and the selectivity of interaction of different spin-labelled lipids with the Na,K-ATPase, and to study the dynamics of the protein-associated lipids (Marsh, 1985; Marsh and Watts, 1982).

The number of lipid association sites is determined by the intramembranous perimeter of the protein and therefore is related directly to the secondary structure and state of assembly of the transmembrane domains (Marsh, 1993). The selectivity of interaction with negatively charged lipids is determined, at least in part, by the presence of basic amino acid side chains in the vicinity of the phospholipid headgroups, again a structurally related parameter (Marsh, 1988). These parameters therefore constrain structural models of the protein, and can be used to check the structural integrity of preparations that have been proteolytically tailored (Esmann et al., 1994).

2. Lipid–protein association determined by spin-labelled lipid probes

The exchange equilibrium for a spin-labelled lipid, L^*, interacting with a membrane protein, P, in a background of unlabelled lipid, L, can be depicted as:

$$L_{N_b}P + L^* \leftrightarrow L_{N_b-1}L^*P + L$$

where N_b is the number of lipid association sites on the protein. In an ESR experiment, the spin-labelled lipid is present at probe concentrations of $\leq$1 mol% relative to membrane lipid. The relative lipid association constant, K_r, for exchange between labelled and unlabelled lipid is given by:

$$K_r = \frac{[L^*P] \cdot [L]}{[LP] \cdot [L^*]} \quad (1)$$

For multiple association sites, the fraction, f, of spin-labelled lipid associated with the protein is given by (Marsh, 1985; Brotherus et al., 1981):

$$\frac{1-f}{f} = \frac{n_t/(N_b - 1)}{K_r} \quad (2)$$

where n_t is the total lipid/protein ratio in the membrane. Hence, both the stoichiometry (N_b) and selectivity (K_r) of interaction of spin-labelled lipids with the Na,K-ATPase may be obtained by quantitating the two-component ESR spectra, using spectral subtraction techniques (cf. Fig. 1).

The spin-label probes that are routinely used to provide the spectral resolution necessary for studying lipid–protein interactions bear a DOXYL nitroxide on the C-14 atom of the lipid chain. However, it is worthwhile to note that a series of different fatty acids, in which the PROXYL nitroxide is incorporated within the backbone of the hydrocarbon chain, also evidence the two-component ESR spectra characteristic of lipid–protein interactions with the Na,K-ATPase (Esmann et al., 1988a). These spin labels belong to the class of minimal perturbation probes, and indicate that specific motional restriction of first-shell lipids associated with Na,K-ATPase is detected irrespective of the nature of the spin-label group.

3. Stoichiometry of lipid–protein interaction

The numbers of motionally restricted lipids per Na,K-ATPase αβ protomer determined by spin-label ESR are given in Table 1. The values are obtained either by lipid–protein titration with reconstituted Na,K-ATPase, or by using a non-selective spin-labelled phospholipid (viz., phosphatidylcholine, for which $K_r \approx 1$) in purified native membranes. Values are also included for prepara-

Table 1
Stoichiometries, N_b, of motionally restricted lipids associated with Na,K-ATPase and Ca-ATPase

Protein	N_b (mol/mol)	Reference
Na,K-ATPase, kidney	31.5 ± 1.5	Brotherus et al. (1981)
Na,K-ATPase, shark	33 ± 3	Esmann et al. (1985)
	30–33	Esmann et al. (1988b)
Na,K-ATPase, shark, trypsinised	30 ± 3	Arora et al. (1998)
Ca-ATPase	22 ± 2	Silvius et al. (1984)
	24 ± 5	Thomas et al. (1982)

All values are given per protein monomer.

tions in which the bulk of the extramembranous sector of the Na,K-ATPase has been removed by extensive trypsinisation.

The stoichiometry of first-shell lipids is determined by the intramembranous structure and assembly of the Na,K-ATPase. For a simple arrangement as in an α-helical sandwich, the number of lipid sites at the intramembranous perimeter is related directly to the number (n_α) of transmembrane helices (Marsh, 1997):

$$N_b = \pi \left(\frac{D_\alpha}{d_{ch}} + 1 \right) + \frac{n_\alpha D_\alpha}{d_{ch}} \quad (3)$$

where D_α is the helix diameter and d_{ch} is the diameter of a lipid chain. For Na,K-ATPase (i.e., $n_\alpha = 11$), Eq. (3) predicts that $N_b = 33$, in reasonable accord with values from experiment. This agreement may be to some extent fortuitous, because it assumes a monomeric protein, whereas some intramembranous surface likely would be inaccessible in a dimer. In addition, the related Ca-ATPase has 10 transmembrane helices, yet its lipid stoichiometry ($N_b = 22$) is considerably smaller than predicted from Eq. (3) ($N_b = 31$). For whatever the reason, there are therefore considerable differences in lipid–protein interactions with these two P-type ATPases.

4. Lipid–protein selectivity

Fig. 2 shows the ESR spectra from different spin-labelled lipid species interacting with the Na,K-ATPase in purified membranes from shark salt gland. The selectivity of lipid association increases from the bottom to the top spectrum, as seen from the intensity of the peaks in the outer wings of the spectrum that arise from the motionally restricted lipid population (cf. Fig. 1).

The association constants of the different spin-labelled lipid species, relative to that for phosphatidylcholine, are given in Table 2. Greatest specificity of interaction is found for anionic lipids, but those with

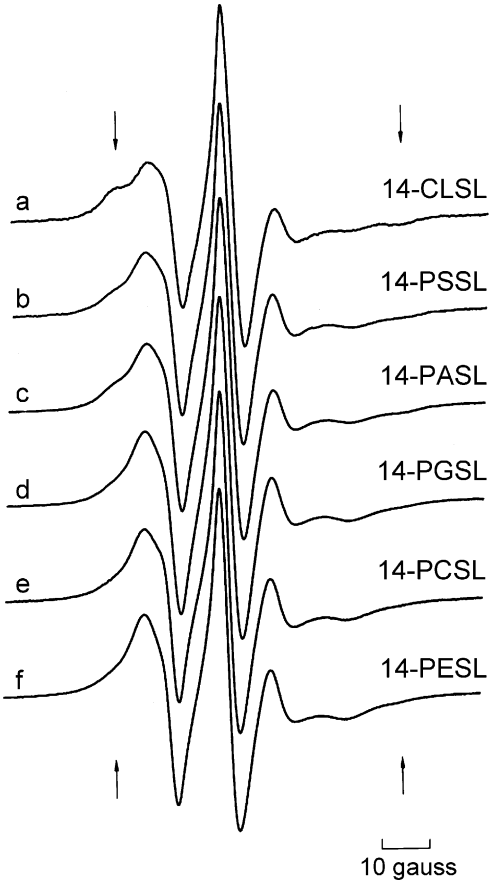

Fig. 2. ESR spectra from *sn*-2 C-14 spin-labelled phospholipids in Na,K-ATPase membranes from *Squalus acanthias*. (a) Ptd_2Gro, 14-CLSL; (b) PtdSer, 14-PSSL; (c) Ptd, 14-PASL; (d) PtdGro, 14-PGSL; (e) PtdCho, 14-PCSL; (f) PtdEtn, 14-PESL. Scan range = 10 mT; $T = 4\,^\circ C$ (Esmann et al., 1985).

the same negative charge do not necessarily display the same selectivity. Of the phospholipids tested, cardiolipin exhibits highest selectivity for the Na,K-ATPase, and phosphatidylserine has the same degree of selectivity as does phosphatidic acid. The selectivity of stearic acid is similar to that of these latter two lipids. Not surprisingly, this pattern of selectivity for the various negatively charged lipids differs from that for other integral proteins (cf. Marsh and Horváth, 1998).

The sialic acid-bearing glycosphingolipids, exemplified by spin-labelled analogues of the gangliosides G_{M1}, G_{M2} and G_{M3}, exhibit no selectivity of interaction with the Na,K-ATPase, relative to phosphatidylcholine (Esmann et al., 1988c). Only the disialoganglioside analogue, G_{D1b}, displays a slight selectivity (see Table 2). Correspondingly, sphingomyelin, which is the sphingolipid analogue of phosphatidylcholine, displays no selectivity relative to the latter glycerolipid (Marsh,

Table 2
Relative association constants (K_r/K_r^{PC}) for the interaction of spin-labelled phospholipids and gangliosides with membranous Na,K-ATPase from *Squalus acanthias*

Phospholipid[a]	K_r/K_r^{PC}	$\Delta G - \Delta G_{PC}$ (kJ/mol)	Ganglioside, etc.[b]	K_r/K_r^{PC}	$\Delta G - \Delta G_{PC}$ (kJ/mol)
Ptd_2Gro	3.8	−3.0	G_{D1b}	1.2	−0.5
Ptd	1.5	−0.9	G_{M1}	1.1	−0.1
PtdSer	1.7	−1.2	G_{M2}	1.0	0.0
PtdGro	0.9	+0.2	G_{M3}	0.9	0.1
PtdEtn	0.9	+0.2	St	1.7	−1.2
PtdCho	1.0	0.0	ASL	0.8	0.5

Data from Esmann et al. (1985, 1988c). All values are referred to spin-labelled phosphatidylcholine; $\Delta G - \Delta G_{PC}$ is the free energy of interaction, relative to phosphatidylcholine.
[a] Ptd_2Gro, cardiolipin; Ptd, phosphatidic acid; PtdSer, phosphatidylserine; PtdGro, phosphatidylglycerol; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine.
[b] St, stearic acid; ASL, androstanol.

1987). Also, the spin-labelled sterol, ASL, which is an androstanol derivative, associates with the Na,K-ATPase but does not display a selectivity greater than that for phosphatidylcholine (see Table 2).

Spin-label ESR experiments are also able to provide information on the origin of the specificity of lipid interaction with the Na,K-ATPase (Horváth et al., 1990; Esmann and Marsh, 1985; Powell et al., 1987). These include investigation of both pH and ionic strength dependences, as well as chemical modification of the lipids.

4.1. Dependence on ionic strength

Fig. 3 shows the ionic screening of the selectivity of interaction of anionic lipids with the Na,K-ATPase.

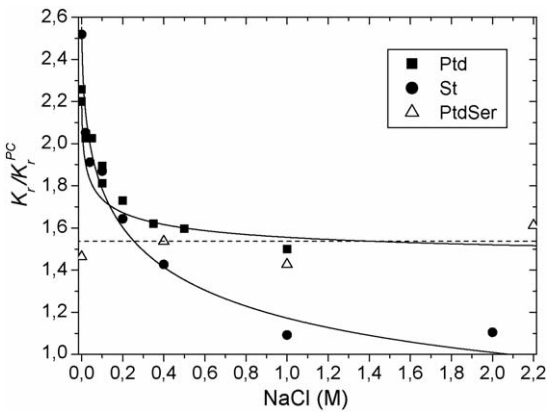

Fig. 3. Ionic strength (I) dependence of the relative association constants (K_r) for spin-labelled phosphatidic acid (squares), stearic acid (circles) and phosphatidylserine (triangles), with Na,K-ATPase. The values are normalised to the relative association constant (K_r^{PC}) for spin-labelled phosphatidylcholine. Solid lines are predictions of Eqs. (4) and (5) from Debye–Hückel theory, with $Z_L = -1$, $Z_P = +1$, $a_P = 3.5$ nm and the parameters given in Table 3. Data from Esmann and Marsh (1985).

Whereas the selectivity for stearic acid and for phosphatidic acid decreases with increasing salt concentration, that for phosphatidylserine is insensitive to salt. Clearly none of the selectivity of phosphatidylserine, relative to that of phosphatidylcholine, is of a simple coulombic origin. Also, high salt concentration does not screen entirely the selectivity of phosphatidic acid relative to phosphatidylcholine, which it does for stearic acid.

The ionic strength dependence of the lipid selectivity shown in Fig. 3 is described, at least qualitatively, by Debye–Hückel theory. The relative association constant in the presence of ions is given by (cf. Eq. (1)):

$$K_r = K_r^0 \left(\frac{\gamma_{L^*} \gamma_P}{\gamma_{L^*P}} \right) \tag{4}$$

where K_r^0 is the relative association constant in the absence of ionic screening, and γ_{L^*}, γ_P and γ_{L^*P} are the activity coefficients of the spin-labelled lipid, protein and lipid–protein complex, respectively (Marsh, 1985). The activity coefficients at ionic strength I are given by (Robinson and Stokes, 1955):

$$\ln \gamma_j = \frac{-Z_j^2 e^2}{8\pi\varepsilon_0 \varepsilon kT} \frac{\kappa}{1 + \kappa a_j} \tag{5}$$

where Z_j is the charge on species j, a_j the interaction distance of species j with counterions and $\kappa = (2000 N_A e^2 I/\varepsilon_0 \varepsilon kT)^{1/2}$ is the inverse Debye screening length. The relative association constants, K_r^0, and parameters obtained from fitting the ionic strength dependence of selectivity for anionic lipids are given in Table 3. The ionic screening of the selectivity for stearic acid is described adequately, with reasonable values for the parameters, suggesting that this selectivity is of simple electrostatic origin. Again, however, it is found that the limited screening for phosphatidic acid does not cor-

Table 3
Relative association constants, K_r^0/K_r(PtdCho), in the absence of salt, and effective ionic interaction distances, a_L, deduced from the ionic strength dependence of the selectivity for interaction of spin-labelled lipids with Na,K-ATPase, according to Eqs. (4) and (5)

Lipid[a]	K_r^0/K_r(PtdCho)	a_L (nm)	$\Delta G - \Delta G_{PC}$ (kJ/mol)[b]
Ptd	2.3	1.0	−1.9
PtdSer	1.5	[c]	−1.0
St	2.7	0.22	−2.3

Relative association constants are referred to that for phosphatidylcholine, K_r (PtdCho). Data from Esmann and Marsh (1985).
[a] Ptd, phosphatidic acid; PtdSer, phosphatidylserine; St, stearic acid.
[b] Free energy of interaction in the absence of ionic screening, relative to phosphatidylcholine.
[c] No ionic strength dependence was found for PtdSer.

respond very well to the simple coulombic model (and completely not for phosphatidylserine).

4.2. Dependence on pH

Fig. 4 shows that the selectivity of stearic acid and phosphatidic acid for interaction with the Na,K-ATPase depends on pH, with a conventional titration according to the Henderson–Hasselbach equation (Marsh, 1995). The relative association constants of the protonated lipid, $K_r(L^*H)$, and of the ionised lipid, $K_r(L^*)$, and also the pK_as at the lipid–protein interface, pK_a^{LP}, are given in Table 4. Protonation of the lipid polar headgroup of stearic acid and phosphatidic acid gives rise to a decrease in relative association constant. Phosphatidylserine, on the other hand, does not titrate in the pH range of the experiment. In the singly protonated states, neither stearic acid nor phosphatidic acid has a greater selectivity for the Na,K-ATPase than does phosphatidylcholine. In the case of phosphatidic acid, the effect of pH titration is greater than that of electrostatic screening, which suggests an additional contribution to the selectivity of interaction of this lipid with the Na,K-ATPase. This most probably comes from hydrational contributions that are reduced on protonation of the lipid, in analogy with the effects of headgroup protonation on lipid bilayer chain-melting phase transitions (Cevc et al., 1980, 1981; Cevc and Marsh, 1987).

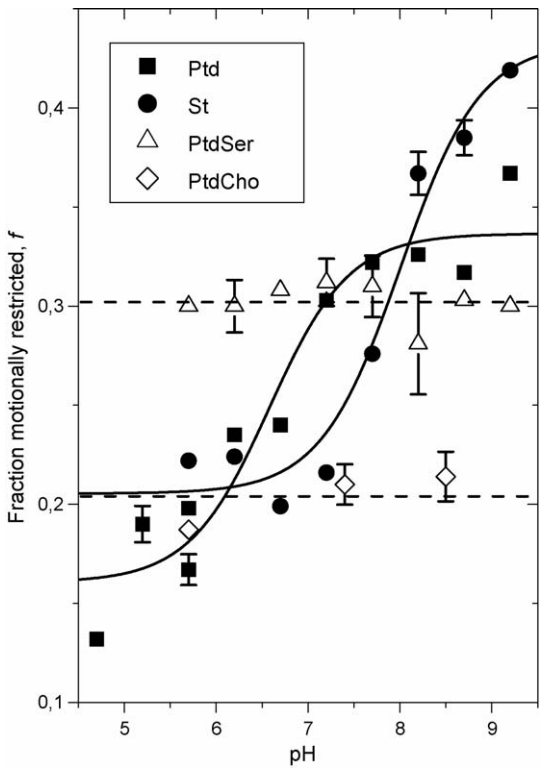

Fig. 4. pH dependence of the fraction (f) of spin-labelled phosphatidic acid (squares), stearic acid (circles) and phosphatidylserine (triangles), associated with Na,K-ATPase. The corresponding values for spin-labelled phosphatidylcholine (diamonds) are also given. Solid lines are fits of a standard pH titration, yielding the parameters given in Table 4. Data from Esmann and Marsh (1985).

From the cyclic nature of the coupled equilibria for protonation and lipid–protein interaction, the shift in lipid pK_a at the lipid–protein interface is given by (Marsh, 1988, 1995):

$$pK_a^{LP} - pK_a^{L} = \log_{10}\left(\frac{K_r(L^*H)}{K_r(L^*)}\right) \quad (6)$$

where pK_a^L is the pK_a in the bulk membrane lipid environment. The shift in pK_a of phosphatidic acid at the lipid–protein interface is in qualitative accordance with Eq. (6), but stearic acid displays a large shift, in the opposite direction to that predicted for stabilisation of the dissociated acid. In the latter case, some other interaction must outweigh the stabilisation of the ionised form of the fatty acid by the protein. The most likely candidate is a change in polarity associated with a shift in vertical location of the fatty acid on protonation (Rama Krishna and Marsh, 1990; Miyazaki et al., 1992).

Table 4
Relative association constants, $K_r(L^*H)$ and $K_r(L^*)$, of the protonated and ionised forms (L^*H and L^*, respectively) of spin-labelled lipids interacting with Na,K-ATPase, and the pK_as of the lipids, pK_a^{LP} and pK_a^{L}, at the lipid–protein interface (see Fig. 4) and in bilayers, respectively

Lipid[a]	$K_r(L^*H)/K_r$(PtdCho)	$K_r(L^*)/K_r$(PtdCho)	pK_a^{LP}	pK_a^{L}
Ptd	0.7	1.9	6.6	7.4[b]
PtdSer	1.6	–	>9.2	–
St	1.0	2.8	8.0	6.6

Relative association constants are referred to that for phosphatidylcholine, K_r(PtdCho). Data from Esmann and Marsh (1985).
[a] Ptd, phosphatidic acid; PtdSer, phosphatidylserine; St, stearic acid.
[b] Determined in dimyristoyl phosphatidylcholine (Horváth et al., 1988).

Table 5
Association constants relative to spin-labelled phosphatidylcholine, K_r/K_r^{PC}, for spin-labelled lipids interacting with control Na,K-ATPase membranes and membranes trypsinised in the presence of Rb^+ (Rb-tryp.) or of Na^+ (Na-tryp.)

Lipid[a]	Control	Rb-tryp.	Na-tryp.	$\Delta\Delta G$ (kJ/mol)[b]
StH	1.5	1.5	1.5	0.0
St^-	3.5	2.8	2.8	0.5
PtdSer	2.0	1.9	2.0	0.0
PtdCho	1.0	–	–	0.0

Data from Arora et al. (1998).
[a] StH, stearic acid at pH 6.0; St^-, stearic acid at pH 9.0; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine.
[b] $\Delta\Delta G$ is the change in interaction free energy resulting from trypsinisation in Na^+.

4.3. Trypsinised Na,K-ATPase

Extensive trypsinisation of the Na,K-ATPase, which removes a large proportion of the cytoplasmic sector of the protein (Esmann et al., 1994), provides a potential means to identify those parts of the protein sequence that contribute to the lipid selectivity. Values of the relative association constant, K_r/K_r^{PC}, for different spin-labelled

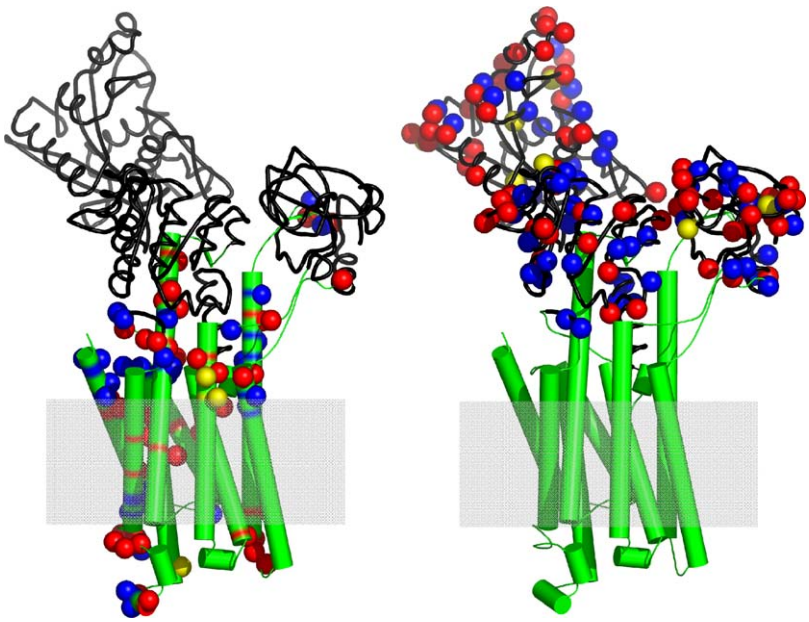

Fig. 5. Assumed transmembrane topography of the Na,K-ATPase α-subunit using the Ca-ATPase crystal structure (Toyoshima et al., 2000) as template for sequence alignments. The fragments that remain in the membrane after trypsinisation in the presence of Rb^+ are given in green, and the parts removed by trypsin are given in black. Charged residues in the tryptic fragments (left-hand side), or in the parts removed by trypsin (right-hand side) are indicated by blue (positive) and red (negative) spheres. Histidine residues are indicated by yellow spheres. The putative location of the hydrophobic core of the membrane is given in grey. Adapted from Esmann et al. (2006). This figure was prepared using PyMOL (Delano, 2002).

lipids in control and trypsinised Na,K-ATPase preparations are given in Table 5. For a given spin-labelled lipid, the relative affinities are of similar magnitudes in control and trypsinised preparations. This implies not only that the intramembranous surface of the Na,K-ATPase remains largely intact (cf. Table 1), but also that those regions of the protein at the membrane surface which contribute to the lipid selectivity are largely preserved on extensive trypsinisation. Only in the case of stearic acid in the fully ionised form, which is the lipid that shows the greatest selectivity for Na,K-ATPase, are significant differences in selectivity observed between the control

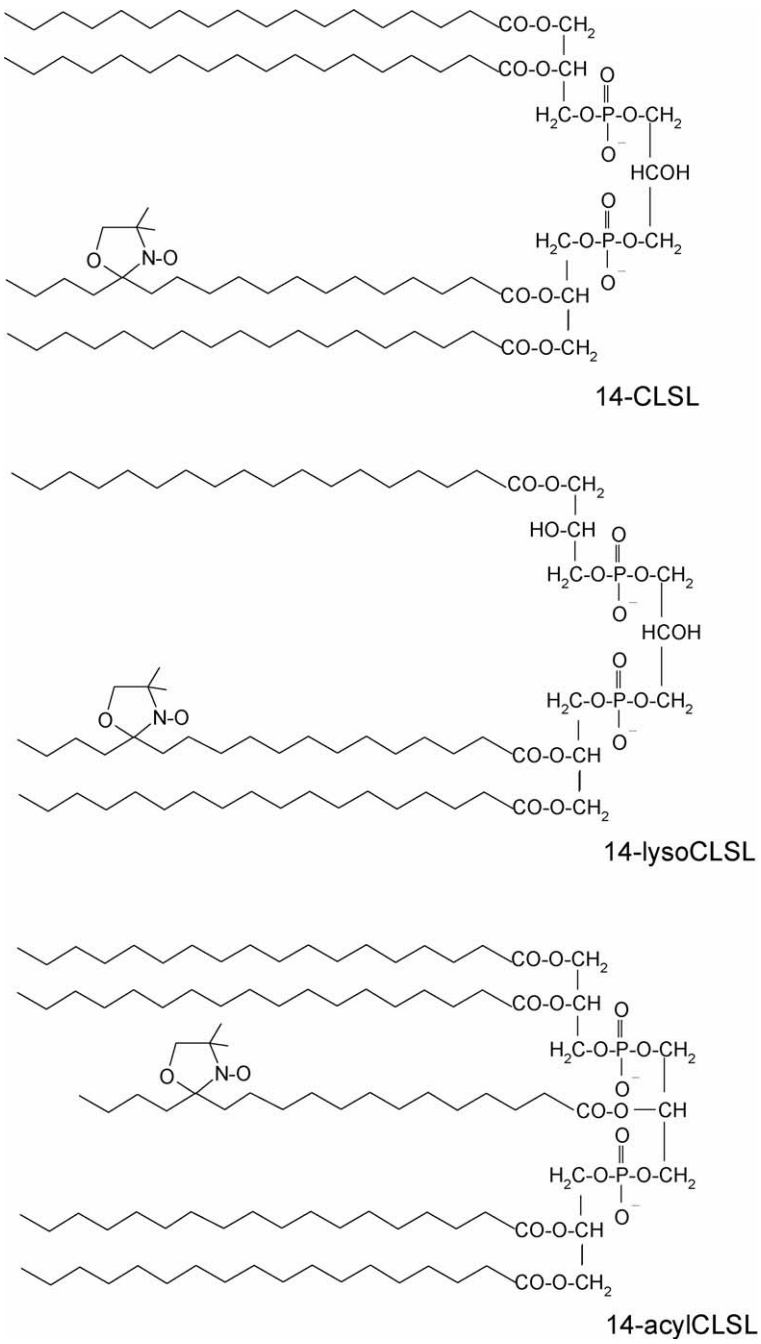

Fig. 6. Structures of spin-labelled diphosphatidylglycerol derivatives. From top to bottom: cardiolipin, 14-CLSL; lysocardiolipin, 14-lysoCLSL; acyl cardiolipin, 14-acylCLSL.

Table 6
Association constants, K_r/K_r^{PC}, referred to spin-labelled phosphatidylcholine, for different spin-labelled cardiolipin analogues interacting with Na,K-ATPase membranes from *Squalus acanthias*, in the presence and absence of 0.1 M NaCl

Lipid[a]	0.0 M NaCl		0.1 M NaCl	
	K_r/K_r^{PC}	$\Delta\Delta G$ (kJ/mol)[b]	K_r/K_r^{PC}	$\Delta\Delta G$ (kJ/mol)[b]
14-CLSL	5.8	0.0	3.1	0.0
14-lysoCLSL	7.2	−0.5	3.0	0.0
14-acylCLSL	4.9	+0.4	2.7	+0.3

Data from Esmann et al. (1988d).
[a] 14-CLSL, spin-labelled cardiolipin (Ptd_2Gro); 14-lysoCLSL, *sn*-2 lysocardiolipin; 14-acylCLSL, *sn*-2′ *O*-acyl cardiolipin.
[b] $\Delta\Delta G$ is the change in interaction free energy resulting from chemical modification of (tetraacyl) cardiolipin.

and trypsinised membranes. In this case, fine structural changes and the removal of certain critical groups must contribute to modulation of the selectivity for ionised stearic acid. Near constancy of the other lipid selectivities suggests, however, that the primary determinants of selectivity for the Na,K-ATPase are preserved in the limit membranes produced by extensive trypsinisation.

Assembly of the transmembrane segments of the Na,K-ATPase, and in particular the intramembranous perimeter of the protein, therefore, is largely preserved on treatment with trypsin. Retention of much of the native lipid specificity suggests that most of the protein residues responsible reside in the membrane-bound tryptic fragments (see Fig. 5), and that the arrangement and orientation of their side chains is largely conserved in the trypsinised preparations. Positive charge on the extracellular side of the membrane is located exclusively in the C-terminal 19-kDa fragment, whereas all four transmembrane tryptic fragments contribute positive charges at or near the cytoplasmic membrane surface (see left-hand side of Fig. 5). These could be sufficient to explain most of the selectivity for negatively charged lipids in native membranes. Positively charged residues in the cytoplasmic part of the α-subunit removed by trypsin that are situated close to the membrane surface (see right-hand side of Fig. 5) could be responsible for the higher selectivity of control membranes for stearic acid at pH 9.0.

4.4. Cardiolipin analogues

The spin-labelled cardiolipin (Ptd_2Gro) analogues illustrated in Fig. 6 can be used to explore further features of the selectivity of lipid interactions with the Na,K-ATPase. Specifically, comparison of cardiolipin with its lyso- and *O*-acyl derivatives – in which the number of fatty acid chains is varied from 3 to 5 – provides essential information on possible contributions of hydrophobic interactions to lipid–protein selectivity.

Table 6 gives the relative association constants for the different cardiolipin analogues in Na,K-ATPase membranes, at two different salt concentrations (Esmann et al., 1988d). The three analogues display rather similar degrees of selectivity and response to salt. Therefore, the greater number of chains does not appear to contribute substantially to the pronounced selectivity of interaction of the tetraacyl phospholipid cardiolipin with Na,K-ATPase (cf. also Table 2). This also means that hydrophobic contributions to the energetics of lipid–protein interaction with Na,K-ATPase are essentially the same as those for lipid–lipid interactions in the fluid bilayer regions of the membrane.

Salt partially screens the strong selectivity for cardiolipin, indicating direct electrostatic contributions to the interaction of this lipid with Na,K-ATPase. However, complete removal of the electrostatic charge by methylating the headgroup phosphates does not reduce the cardiolipin selectivity to appreciably less than that in 0.1 M NaCl (Esmann et al., 1988d). As for phosphatidic acid, there are very significant non-electrostatic contributions to the selectivity of interaction of cardiolipin with Na,K-ATPase.

5. Lipid exchange dynamics

Exchange between lipids at the protein interface and those in the bulk of the membrane is a significant dynamic feature of the lipid–protein interactions with the Na,K-ATPase (Arora et al., 1999). The exchange process is characterised by the on- and off-rate constants τ_f^{-1} and τ_b^{-1}, respectively:

$$L^* + LP \underset{\tau_b^{-1}}{\overset{\tau_f^{-1}}{\longleftrightarrow}} L^*P + L$$

for interaction of spin-labelled lipid L^* with the protein. At equilibrium, material balance relates the two rate con-

Table 7
Lipid exchange off-rate constants, τ_b^{-1}, for spin-labelled stearic acid in native Na,K-ATPase membranes from *Squalus acanthias* at 25 °C, and in control membranes and those trypsinised in the presence of Rb^+ or Na^+

Membrane[a]	$K_r(L^*)/K_r(L^*H)$	τ_b^{-1} (L^*H) (s^{-1})[a]	τ_b^{-1} (L^*) (s^{-1})[a]
Native	2.6	4.1×10^6	1.6×10^6
Control	2.3	1.6×10^6	0.7×10^6
Rb-tryps.	1.8	0.9×10^6	0.5×10^6
Na-tryps.	1.9	0.7×10^6	0.5×10^6

Values are presented for protonated stearic acid (L^*H) at pH 6.0, and for ionised stearic acid (L^*) at pH 9.0. Ratio of association constants, $K_r(L^*)/K_r(L^*H)$, of ionised and protonated forms is also given. Data from Arora et al. (1999).
[a] Taking a value of $T_{1,f}^0 = 0.44\,\mu s$.

stants to the respective populations:

$$(1-f)\tau_f^{-1} = f\tau_b^{-1} \tag{7}$$

where f is the fraction of lipid that is associated with the protein. The quantity of interest is the off-rate, τ_b^{-1}. This represents the intrinsic rate for one-to-one exchange at the lipid–protein interface, whereas the on-rate is diffusion controlled and its effective value τ_f^{-1} is dependent on the relative population, $(1-f)/f$, of fluid lipids.

Because the lipid exchange rates are slow, relative to the dynamic sensitivity of the spin-label lineshapes, it is necessary to exploit the saturation properties of the ESR spectrum (see Marsh et al., 1998). These are determined by the nitroxide spin-lattice relaxation time (T_1) that lies typically in the microsecond time regime. The increase in spin-lattice relaxation rate of the protein-interacting lipids that is caused by exchange with the fluid lipid population is given by (Horváth et al., 1993):

$$\frac{1}{T_{1,b}} - \frac{1}{T_{1,b}^0} = \frac{\tau_b^{-1}}{1 + T_{1,f}^0\tau_f^{-1}} \tag{8}$$

where $T_{1,b}^0$ and $T_{1,f}^0$ are the spin-lattice relaxation times of the motionally restricted and fluid spin-labelled lipids, respectively, in the absence of exchange. It is thus possible to determine the intrinsic off-rates, τ_b^{-1}, from progressive saturation ESR measurements (Arora et al., 1999). Table 7 gives values for the exchange rates of both protonated (L^*H) and charged (L^*) forms of stearic acid in native Na,K-ATPase membranes. Data are also included for membranes extensively trypsinised in the presence of either Rb^+ or Na^+, together with controls.

The results in Table 7 indicate that lipids at the intramembranous surface of the Na,K-ATPase exchange continuously with those in the bulk regions of the membrane, on the spin-lattice relaxation timescale of spin-labelled lipids, i.e., in the microsecond regime. Lipid exchange rates in the trypsinised membranes are not very much slower than those in the corresponding control membranes (see Table 7). They are, however, considerably slower than those in native membranes. This difference, in common with the controls, is attributed to time- and temperature-dependent changes taking place in the membranes during the incubation procedure needed for trypsinisation (cf. Esmann et al., 1987).

Exchange rates are faster for the protonated fatty acid than for the ionised fatty acid, in all cases (see Table 7). This directly reflects the selectivity of the ionised over the protonated species in association of fatty acids with the Na,K-ATPase (cf. Table 4). From Eqs. (2) and (7), an inverse relation is obtained between the lipid off-rates and association constants (see Marsh, 1990):

$$\frac{\tau_b^{-1}(L^*H)}{\tau_b^{-1}(L^*)} = \frac{K_r(L^*)}{K_r(L^*H)} \tag{9}$$

which is consistent with the data presented in Table 7. From the complete range of ESR studies in Arora et al. (1999), it is found that the lipid chains directly contacting the Na,K-ATPase have temperature-dependent, submicrosecond segmental motions, and that these first-shell lipid molecules exchange with the bulk membrane lipids on the microsecond timescale. These essential features of the dynamics of the lipid–protein interaction with the Na,K-ATPase are largely preserved on removal of the extramembranous portions of the protein by extensive trypsinisation.

6. Conclusion

This review has concentrated on the fascinating biophysical aspects of lipid–protein interactions with the Na,K-ATPase. Nevertheless, these results are not without their functional correlates. Lipid dynamics are essential to enzymatic function (Johannsson et al., 1981; Cornelius, 2001). Anionic phospholipids are necessary for efficient reconstitution (Cornelius and Skou, 1984). A minimum number of phospholipids must be retained to support full activity of the solubilised enzyme (Esmann, 1984). Free fatty acids, which display a selectivity for

the Na,K-ATPase (see Table 2), which also specifically inhibit the enzyme (Ahmed and Thomas, 1971), whereas phosphatidylserine stabilises the solubilised enzyme (Hayashi et al., 1989; Shinji et al., 2003).

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