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Rapid report

Selectivity of lipid–protein interactions with trypsinized Na,K-ATPase studied by spin-label EPR

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Abstract

The selectivity of the lipid–protein interactions in trypsinised Na,K-ATPase membranes from *Squalus acanthias* has been determined by using EPR spectroscopy with different lipid probes spin-labelled on the 14-C atom of the fatty acid chain. From measurements at low ionic strength and different pH values, the pattern of selectivity is: $(\text{stearic acid})^- > (\text{phosphatidylserine})^- > (\text{stearic acid})^0 > (\text{phosphatidylcholine})^\pm$, where superscripts indicate the formal electrostatic charge on the lipid headgroup. This is in the same order as that determined with native Na,K-ATPase membranes [M. Esmann, D. Marsh, Biochemistry 24 (1985) 3572–3578]. The selectivity for phosphatidylserine is independent of pH, over the range pH 6.0–9.0, as found also for native membranes. For membranes trypsinised in the presence of Rb^+ ions, and in the presence of Na^+ (which allows more extensive proteolysis), the relative association constants, K_r, of all lipids are the same as for control membranes, with the exception of ionised $(\text{stearic acid})^-$ that shows the highest specificity. Therefore, both the stoichiometry and the principal determinants of the specificity of lipid–protein interaction are preserved on extensive trypsinisation of Na,K-ATPase membranes. This has implications for the location and arrangement of those amino acid side chains that determine the lipid selectivity of the native Na,K-ATPase.

Keywords: Na,K-ATPase; Spin label; EPR; Lipid–protein interaction; Lipid selectivity; Transmembrane segment

As an aid to structural and structure–functional studies, membranous Na,K-ATPase has been extensively trypsinised to remove the extramembranous section [1]. On trypsinisation in the presence of Rb^+ ions, the intramembranous section of the α-subunit of the Na,K-ATPase from *Squalus acanthias* is split into several short peptides that could constitute double transmembrane segments and a 19-kDa fragment that could constitute a transmembrane four-helix bundle [2,3]. N-terminal sequencing reveals a high degree of identity and homology of the *S. acanthias* protein with the mammalian kidney enzyme. On trypsinisation in the presence of Na^+ ions, proteolysis of the *S. acanthias* enzyme is more extensive and the 19-kDa fragment, which is essential for retention of Rb^+-occlusion capacity, is no longer preserved.

Previously, we have studied rotational diffusion and overall lipid–protein interactions of the trypsinised Na,K-ATPase from *S. acanthias* [3]. These results suggest that the intramembranous assembly of the protein is largely preserved on trypsinisation in the presence of Rb^+, which is a prerequisite for detailed structural studies. Also, we have characterised the secondary structure by infrared spectroscopy, providing evidence that the intramembra-

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nous section of the protein may have considerable β-sheet content [4]. In the present work, we look in detail at the specificity of lipid interactions with the trypsinised preparations of membranous Na,K-ATPase by using EPR spectroscopy and spin-labelled lipids. Both the intramembranous perimeter and the major determinants of the lipid selectivity of the native Na,K-ATPase appear to be retained in the trypsinised preparations.

Na,K-ATPase from the rectal gland of *S. acanthias* was prepared as described previously [5], but omitting the treatment with saponin. The Na,K-ATPase constituted typically 70% of the total protein (determined as the content of α- and β-subunits from SDS gel electrophoresis), and the specific activity was 1400–1700 μmol of ATP hydrolysed mg^{-1} of protein h^{-1}. Na,K-ATPase activity and protein content were determined as previously described [6]. Membrane lipids were extracted with $CHCl_3/CH_3OH$ (2:1, v/v). Trypsinisation of Na,K-ATPase membranes was carried out essentially as described in Ref. [2]. The membranes were washed by centrifugation 3 times in a buffer containing 10 mM RbCl or NaCl, 15 mM histidine, 1 mM CDTA (pH 7.0 at 20°C), and 25% glycerol. Samples were stored at −20°C in this buffer. Control enzyme was treated as above, but omitting trypsin. Trypsin, trypsin inhibitor, histidine and Tris were obtained from Sigma (St. Louis, MO).

Stearic acid (14-SASL), phosphatidylserine (14-PSSL) and phosphatidylcholine (14-PCSL) spin-labelled on the 14-C atom of the (*sn*-2) chain were prepared as described in Ref. [7]. Two milligram of Na,K-ATPase (native or trypsinised) protein in 10 ml of either histidine buffer (10 mM, 1 mM EDTA, pH 6.0) or Tris buffer (10 mM, 1 mM EDTA, pH 9.0) were incubated at 23°C for 1 h with 40 μg of spin-labelled lipid, added as an ethanol solution (10 mg/ml). Membranes were pelleted by centrifugation at 45,000 rpm for 45 min at 4°C. The pellet was taken up into a 1-mm diameter glass capillary and trimmed to a sample length of 5 mm. Lipid dispersions were labelled by mixing the spin-label with extracted membrane lipids in (2:1, v/v) $CHCl_3$:CH_3OH prior to removal of the organic solvent and dispersion of the lipid in buffer. ESR spectra were recorded on a Varian Century Line 9-GHz spectrometer equipped with nitrogen gas-flow temperature regulation. Further details of the ESR spectral analysis and spin-labelling techniques are given in Ref. [8].

The EPR spectra of spin-labelled stearic acid (14-SASL) and phosphatidylserine (14-PSSL) in Na,K-ATPase membranes that have been trypsinised in Rb^+- or Na^+-containing buffer are given in Fig. 1,

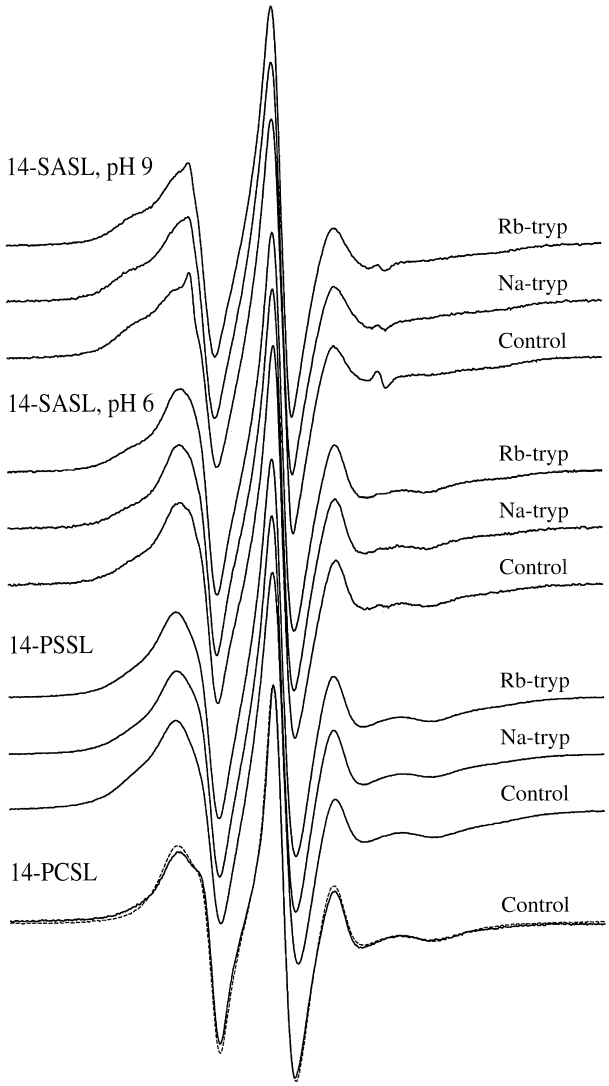

Fig. 1. EPR spectra (from upper to lower groups) of spin-labelled stearic acid (14-SASL), phosphatidylserine (14-PSSL) and phosphatidylcholine (14-PCSL) in Na,K-ATPase membranes trypsinised in Rb^+ (Rb-tryp.), trypsinised in Na^+ (Na-tryp.), or non-proteolysed (control), at 4°C. For 14-SASL, the membranes were suspended in buffer at pH 9.0 (upper group) or at pH 6.0 (lower group), and in all other cases at pH 6.0. The dashed line is the spectrum of 14-PCSL in an aqueous dispersion of the extracted membrane lipids at 4°C. Total scan width = 100 Gauss.

together with those for control samples that were similarly preincubated, but in the absence of trypsin. The EPR spectrum of spin-labelled phosphatidylcholine (14-PCSL) in a control membrane sample and in a dispersion of the extracted membrane lipids (dashed line) is also included in the figure, as a reference. For the 14-SASL spin label, EPR spectra are given for membranes in buffers at pH 9.0 and 6.0, which correspond to fully ionised and fully protonated membrane-bound stearic acid, respectively (e.g., see Ref. [9]).

Comparison of the 14-PCSL EPR spectrum from dispersions of the extracted lipids with that from control membranes indicates the presence of a motionally restricted lipid population, the spectrum of which is resolved in the outer wings of the spectrum of the fluid lipid component, particularly at low field (cf. Ref. [8]). The spectrum of this motionally restricted lipid component is much more marked in the spectra of the 14-SASL and 14-PSSL spin-labelled lipids, and is attributed to those spin-labelled lipids that are interacting directly with the intramembranous section of the protein (see Refs. [8,10]). The greater relative intensity of the motionally restricted lipid spectral component indicates a selectivity of stearic acid and phosphatidylserine for interaction with the trypsinised Na,K-ATPase, as found previously for the native protein [9].

Quantitative determination of the selectivity of the different lipids for the different membranous protein preparations is performed by spectral subtraction [11]. This has been done by using the single-component EPR spectra of the corresponding labels in dispersions of the extracted membrane lipids to match those of the fluid lipid component, and spectra from the gel-phase of sonicated dimyristoyl phosphatidylcholine vesicles to match those of the motionally restricted lipid component, in the Na,K-ATPase membranes. The fractions, f, of motionally restricted lipids that are present in the various membrane preparations, as monitored by the different spin-labelled lipids, are given in Table 1. The largest fraction of motionally restricted spin-labelled lipid in the control membranes is found for stearic acid in the negatively charged form at pH 9.0. The next largest value of f is for phosphatidylserine, for which very similar results are obtained at both pH 9.0 and pH 6.0. The latter correlates with the lack of pH dependence of the selectivity from this lipid that has been found previously in native Na,K-ATPase membranes [9]. The protonated form of stearic acid exhibits a smaller fraction of motionally restricted lipid, but still considerably greater than that for the zwitterionic lipid, phosphatidylcholine, again mirroring the results obtained previously in native Na,K-ATPase membranes [9].

Table 1
Fraction of motionally restricted lipid spin label, f, and association constant relative to spin-labelled phosphatidylcholine, K_r/K_r^{PC}, for stearic acid (14-SASL) at pH 6.0 and pH 9.0, phosphatidylserine (14-PSSL), and phosphatidylcholine (14-PCSL) interacting with control Na,K-ATPase membranes and membranes trypsinized in the presence of Rb^+ (Rb-tryp.) or of Na^+ (Na-tryp.)

Membrane	lipid label	f	K_r/K_r^{PC}
Control	14-SASL, pH 6	0.26	1.5
Rb-tryp.		0.26	1.5
Na-tryp.		0.26	1.5
Control	14-SASL, pH 9	0.45	3.5
Rb-tryp.		0.39	2.8
Na-tryp.		0.40	2.8
Control	14-PSSL	0.32	2.0
Rb-tryp.		0.31	1.9
Na-tryp.		0.32	2.0
Control	14-PCSL	0.19	1.0

Values of f were obtained by spectral subtraction for membranes at 4°C and values of K_r/K_r^{PC} were obtained from Eq. (1).

The association constants of the different lipids, relative to phosphatidylcholine, can be obtained from the equation for equilibrium lipid–protein exchange association for samples of identical total lipid/protein ratio [12,13]:

$$\frac{(1-f)/f}{[(1-f)/f]^{PC}} = \frac{K_r^{PC}}{K_r} \quad (1)$$

where the superscripts PC refer to the 14-PCSL lipid spin label. Values of K_r/K_r^{PC} calculated in this way for the different spin-labelled lipids are given in Table 1. It is noteworthy that the values of the relative affinities K_r/K_r^{PC} for the control and different trypsinised preparations are of a similar magnitude, for a given spin-labelled lipid. This non-trivial result implies both that the intramembranous surface

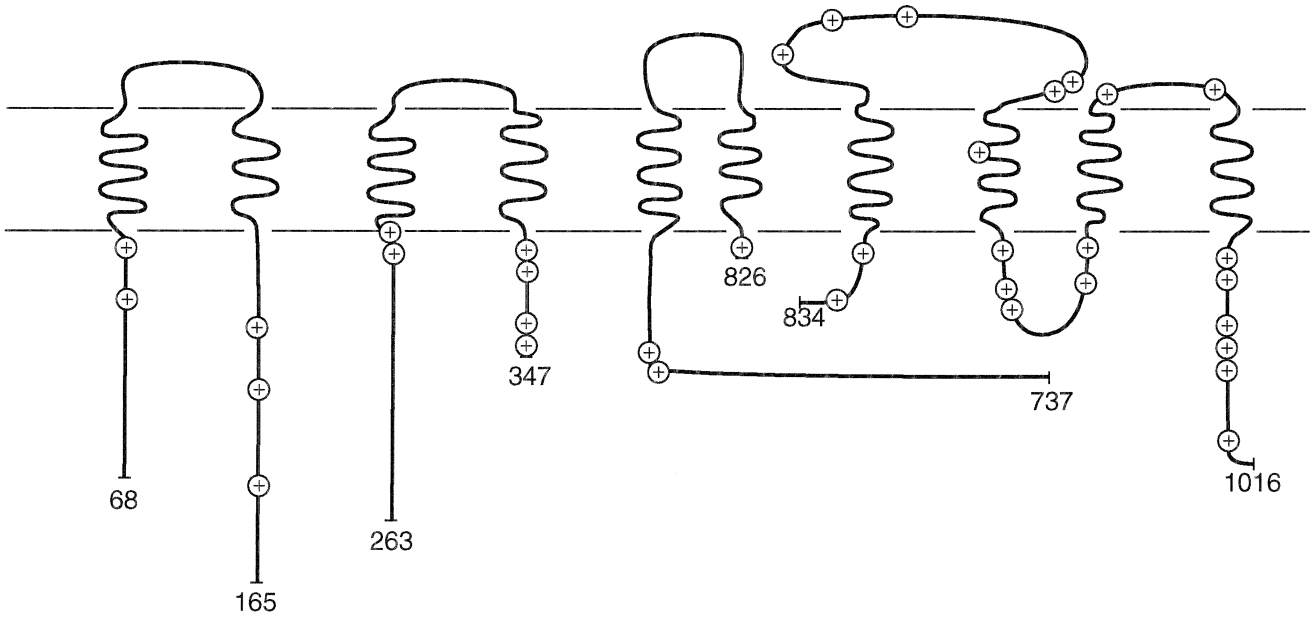

Fig. 2. Putative transmembrane disposition of the tryptic fragments from the Na,K-ATPase α-subunit, with sequence location of positively charged residues (Lys, Arg and His). Adapted from Ref. [14]. Residue numbering corresponds to the sheep α-subunit sequence [15]. *N*-terminal sequences have been determined for the *S. acanthias* α-subunit, and the corresponding C-terminal residues are identified from SDS gel molecular weights and candidate tryptic cleavage sites.

of the Na,K-ATPase remains largely intact on trypsinisation under the conditions used here, and also that those regions of the protein at the membrane surface that contribute to the lipid selectivity are mostly preserved on extensive trypsinisation. Only in the case of the lipid that shows the greatest selectivity for the Na,K-ATPase, viz. 14-SASL in the fully ionised form, are significant differences in selectivity observed between the control and trypsinised membranes. In this latter case, fine structural changes and the removal of certain critical groups presumably contributes to a modulation of the selectivity for ionised stearic acid. The near constancy of the other lipid selectivities, however, suggests both that the stoichiometry of lipid–protein interactions and the primary determinants of the lipid selectivity for the Na,K-ATPase are preserved in the limit membranes produced by extensive trypsinisation.

As discussed previously [3], these new spin label EPR results strongly suggest that the assembly of the intramembranous segments of the Na,K-ATPase, and in particular the intramembranous perimeter of the protein, is largely conserved on treatment with trypsin. The retention of the native lipid specificity suggests further that the protein residues responsible for this reside in the membrane-bound tryptic fragments (see Fig. 2), and that the arrangement and orientation of their side chains is largely preserved in the trypsinised preparations. The positive charges on the extracellular leaflet of the membrane are located exclusively in the C-terminal 19 kDa fragment, whereas all four transmembrane tryptic fragments contain positive charges at or near the membrane surface. These could be sufficient to explain most of the selectivity for negatively charged lipids that is observed with native membranes. Positively charged residues that are remote from the membrane surface in the linear sequence might be situated closer to the surface in the native protein fold, which possibly could account for the higher selectivity of control membranes for 14-SASL at pH 9.0.

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