Structural Integrity of the Membrane Domains in Extensively Trypsinized Na,K-ATPase from Shark Rectal Glands†

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ABSTRACT: Removal of extramembranous portions of the integral membrane protein Na,K-ATPase from shark salt glands by trypsin in the presence of Rb^+ (a K^+ congener) preserves the intramembranous association of the remaining membrane-spanning tryptic peptides. This is evidenced from comparison of the rotational mobility of native and trypsinized Na,K-ATPase using saturation transfer electron spin resonance spectroscopy (ESR) and from study of the lipid–protein interactions using conventional ESR spectroscopy. The interface between the lipids and the intramembranous domains is conserved on removal of the extramembranous parts of the protein, since the population of motionally restricted boundary lipids remains essentially the same in the native and trypsinized preparations. The ability to occlude Rb^+ is also retained by the trypsinized membranes, as previously observed with pig kidney Na,K-ATPase. A 19-kDa fragment remaining when Na,K-ATPase is trypsinized in the presence of Rb^+ is degraded further when the trypsinization is carried out in the presence of Na^+ instead of Rb^+. The rotational mobility of the tryptic fragments in the Na^+-trypsinized membranes is lower than for the Rb^+-trypsinized membranes, indicating rearrangement of the peptides. In addition, occlusion capacity is lost when trypsinization is carried out in Na^+, suggesting a correlation between structure and function in the trypsinized membranes. The sequences of four membrane-spanning tryptic fragments of shark Na,K-ATPase are found to be almost identical to corresponding sequences in pig kidney Na,K-ATPase.

The structural organization of integral membrane proteins depends both on the assembly of the intramembranous domains and on the extramembranous arrangement of the sections of the protein that are exposed to the aqueous phase. Studies on proteolytically cleaved bacteriorhodopsin, a predominantly hydrophobic protein most of the mass of which is confined within the membrane, have indicated that isolated transmembrane fragments are capable of spontaneous reassembly within the lipid milieu (Kahn & Engelman, 1992). Functional reassociation of complementary protein fragments has also been observed in other cases [for a review, see Lemmon & Engelman (1992)]. The question still remains, however, to what extent the membrane-embedded part of an intrinsic membrane protein retain its structure after the extramembranous part has been removed. Such considerations are crucial to experiments involving the removal of the extramembranous domains of membrane enzymes by extensive proteolysis (Karlish et al., 1990) and to the functional relevance of reconstitution experiments with synthetic peptides corresponding to putative transmembrane sequences (Montal, 1990).

The integral membrane transport enzyme examined here is the Na,K-ATPase (EC 3.6.1.37), which has 8 or 10 transmembrane helices in the 112-kDa α-subunit.[1] The function of this enzyme is to transport Na^+ and K^+ across the cell membrane at the expense of ATP hydrolysis (Glynn, 1985; Lingrell et al., 1990). A large part of the α-subunit, about 60%, can be removed from the membrane by treatment with trypsin in the presence of RbCl (Karlish et al., 1990). The segments removed include a large, almost 400 residue long cytoplasmic portion of the protein that bears the nucleotide binding and phosphorylation sites. The remaining membrane-embedded tryptic fragments are still able to occlude cations, which is an essential step in the catalytic cycle of the Na,K-ATPase. The tryptic fragments remaining in the membrane have molecular masses in the range 8–12 kDa, in addition to a larger 19-kDa fragment, and the amino acid sequences of these fragments indicate that they include all the hydrophobic membrane-spanning segments of the protein (Capasso et al., 1992).

The structural integrity of the trypsinized Na,K-ATPase is probed here by rotational diffusion measurements with the spin-labeled protein and studies on the lipid–protein interactions with spin-labeled lipids. Na,K-ATPase is spin-labeled on sulfhydryl groups, and the rotational mobilities of the control enzyme and the trypsinized enzyme are compared by using saturation transfer electron spin resonance spectroscopy

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[1] Abbreviations: α, the 112-kDa catalytic subunit of the Na,K-ATPase; β, the 36 kDa glycosylated subunit of the Na,K-ATPase; CDTA, *trans*-1,2-cyclohexylenedinitrilotetraacetic acid; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; 14-SASL, 14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; 5-MeCl-HgMSL, *trans*-3-methoxycarbonyl-4-(4′-chloromercuribenzamido)methyl-1-oxyl-2,2,5,5-tetramethylpyrrolidine; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate.

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(STESR) (Marsh, 1981). It is found that the trypsinization procedure does not change the state of assembly of the membrane-spanning part of the protein to an appreciable extent. In addition, it is shown that trypsinization under conditions where the 19-kDa fragment is degraded leads to rearrangement of membrane particles and loss of the ability to occlude cations.

The lipid–protein interaction at the interface between lipid and the putative membrane-spanning α-helices is studied with spin-labeled stearic acid in conjunction with conventional ESR spectroscopy (Marsh, 1985). It is found that the number of lipid molecules associated at the intramembranous perimeter of the protein (the boundary layer of lipids) is not affected by trypsinization, i.e., this structural feature of the native protein is preserved and is not affected by removal of large extramembranous sections of the protein.

The Na,K-ATPase preparation used in the present experiments is derived from the rectal glands of the spiny dogfish (*Squalus acanthias*). The amino acid sequence of Na,K-ATPase from this preparation is not known, but it is shown here that three fragments produced upon trypsinization, additionally to the 19-kDa fragment (Esmann & Sottrup-Jensen, 1992), have amino acid sequences almost identical to those of the *Torpedo californica* and pig kidney Na,K-ATPases.

MATERIALS AND METHODS

Preparation of Shark Rectal Gland Na,K-ATPase. Na,K-ATPase from the rectal gland of *S. acanthias* was prepared as described previously (Skou & Esmann, 1979), 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 hydrolyzed/mg of protein per h. Na,K-ATPase activity and protein content were determined as previously described (Esmann, 1988). Lipids were extracted from the membranes as described previously (Esmann et al., 1985).

Covalent Spin-Labeling of Sulfhydryl Groups. Prelabeling of Na,K-ATPase with NEM to block class I SH-groups and sulfhydryl groups of non-Na,K-ATPase proteins in the membrane preparations was performed as follows [see Esmann et al. (1993) for details]: Na,K-ATPase (approximately 1 mg/mL) was incubated at 23 °C with 0.1 mM NEM in 30 mM histidine (pH 7.0 at 23 °C), 5 mM CDTA, 150 mM KCl, and 36% (v/v) glycerol for 60 min. The reaction was stopped by addition of 1 mM 2-mercaptoethanol, and the membranes were washed by centrifugation in 20 mM histidine (pH 7.0 at 20 °C) and 25% (v/v) glycerol at 200000*g*. Three centrifugations in 27-mL tubes were sufficient to remove residual reaction medium. The prelabeled enzyme was stored in 20 mM histidine and 25% (v/v) glycerol at –20 °C. Selective spin-labeling of SH-groups that are essential for the overall Na,K-ATPase activity was performed as follows (Esmann et al., 1993): prelabeled Na,K-ATPase (see above) was incubated for 30 min with 6 μM 5-MeClHgMSL at 23 °C in 30 mM histidine (pH 7.4 at 37 °C) in the presence of 150 mM KCl and 5 mM CDTA. The reaction was stopped by addition of 1 mM 2-mercaptoethanol. The membranes were washed by centrifugation in 20 mM histidine (pH 7.0 at 20 °C) and 25% (v/v) glycerol at 200000*g*. The spin-labeled enzyme was stored in 20 mM histidine and 25% (v/v) glycerol at –20 °C. The spin-label was added as a dimethylformamide (DMF) solution. The final DMF concentration (before washing) was less than 1%.

Trypsinization of Na,K-ATPase. Na,K-ATPase membranes, either native or spin-labeled, were incubated at a concentration of 0.9 mg/mL with 10 mM RbCl or NaCl, 15 mM histidine, and 1 mM CDTA (pH 7.0 at 20 °C) with trypsin (final concentration 0.5 mg/mL). After 60 min at 23 °C a 10-fold excess by weight of trypsin inhibitor was added, in the same buffer, and the sample was allowed to stand for 10 min at 23 °C. The membranes were washed by centrifugation three 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 (Esmann & Sottrup-Jensen, 1992). Samples were stored at –20 °C in this buffer. Control enzyme was treated as above, omitting trypsin.

Rb^+-Occlusion Assay. Occlusion of $^{86}Rb^+$ was assayed using the cation-exchange method previously described (Esmann & Sottrup-Jensen, 1992). It was found that the occlusion capacity was retained when trypsinization was performed in RbCl (Esmann & Sottrup-Jensen, 1992), whereas no Rb^+-occlusion was detected when trypsinization was carried out in NaCl (data not shown). Spin-labeled Na,K-ATPase retained the ability to occlude Rb^+ with a slightly lower affinity for Rb^+ (not shown). Trypsinization of spin-labeled Na,K-ATPase in RbCl or NaCl yielded digestion patterns identical to those of non-spin-labeled Na,K-ATPase (Figure 3), and the ability to occlude Rb^+ after trypsinization was retained only in the membranes trypsinized in the presence of Rb^+ (not shown).

Separation of Peptides by Size-Exclusion HPLC. Two columns packed with TSK-G3000SW (30 cm with 7.5-mm internal diameter) were used in series as described earlier (Capasso et al., 1992). Delipidated trypsinized enzyme was centrifuged, dried under nitrogen, and redissolved in 2% SDS at a concentration of approximately 3 mg of protein/mL. Undissolved material was removed by centrifugation for 20 min in a Beckman Airfuge, and about 1 mg of protein was injected onto the HPLC columns. Protein was eluted at a flow rate of 0.2 mL/min in a sodium acetate buffer (100 mM, pH 4.5) containing 0.5% SDS, and fractions were collected manually.

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed according to the method of Schagger & von Jagow (1987) using 1-mm 16.5% gels. Samples were delipidated before electrophoresis as described earlier (Capasso et al., 1992). To one volume of protein (in SDS solution) was added 4 volumes of methanol at 0 °C. The precipitated protein was centrifuged after incubation for 12 h at –20 °C, and the pellet was dissolved in sample buffer, which contained 100 mM sodium phosphate (pH 7.7), 1% 2-mercaptoethanol, 2% SDS, and 36% urea. Samples were heated to 100 °C for 5 min before electrophoresis. Staining was done in a solution of 0.25% Coomassie Blue R250, 40% ethanol, 10% acetic acid for 1 h, and destaining was done in 30% ethanol and 10% acetic acid. Electroblotting was carried out as described by Matsudaira (1987) using a LKB 2051 Midget Multiblot apparatus with Problott PVDF membranes (Applied Biosystems). Only HPLC-grade solvents and Millipore filtered or twice-destilled water were used. The electroblotting buffer (Matsudaira, 1987) contained 0.005% SDS.

Sequenator Analysis. Edman degradations were carried out on a 477A instrument from Applied Biosystems with on-line analysis of the phenylthiohydantoin (PTH) amino acids by reverse-phase HPLC on a 120A liquid chromatograph. The stained electroblotted samples were cut in small pieces and placed in the cross-flow chamber.

ESR Spectroscopy. Samples of covalently labeled Na,K-ATPase for STESR spectroscopy were prepared according to the following protocol (Esmann et al., 1993): 1 mg of spin-labeled protein was diluted in 10 mL of buffer [30 mM histidine (pH 7.4 at 37 °C), 100 mM NaCl or RbCl, and 1 mM CDTA], and the membranes were pelleted by centrifugation at 6 °C for 45 min at 100000*g*. The pellet was freed from excess buffer, taken up into a 1-mm diameter glass capillary and trimmed to a sample length of 5 mm. ESR spectra were recorded on a Varian Century Line 9-GHz spectrometer equipped with nitrogen gas flow temperature regulation and interfaced to an IBM personal computer. Conventional, in-phase, absorption ESR spectra (V_1 display) were recorded with a modulation frequency of 100 kHz and a modulation amplitude of 1.6 G peak-to-peak. STESR spectra were recorded in the second harmonic, 90° out-of-phase, absorption mode (V'_2 display) with a modulation frequency of 50 kHz and a modulation amplitude of 5 G peak-to-peak. Standardized sample geometry and spectrometer settings and calibrations were employed as in the protocol described in Fajer and Marsh (1982) and Hemminga et al. (1984). Integrals of the STESR spectra, normalized with respect to the intensity of the V_1-mode spectra, were evaluated as described in Horváth and Marsh (1983). Calibrations of the diagnostic STESR line height ratios (L″/L and H″/H) and normalized integral intensities, in terms of the rotational correlation times of 5-MSL-labeled haemoglobin, were taken from Horváth and Marsh (1988). Further details of the ESR spectroscopy are given in Esmann et al. (1987). Samples for analysis of lipid–protein interactions with spin-labeled fatty acid (14-SASL) were prepared as follows: 2 mg of Na,K-ATPase protein in 5 mL of 10 mM Tris buffer (pH 5.9) were incubated at 23 °C for 30 min with 10 μg of 14-SASL, added as a ethanol solution. After centrifugation as above, the spin-labeled membranes were taken up into a 1-mm diameter glass capillary. The modulation amplitude was 1.25 G (Esmann et al., 1985).

Materials. Trypsin (T-8642), trypsin inhibitor (T-9128), and molecular weight markers (MW-SDS-17S and MW-SDS-70) were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Separation and Determination of Partial Amino Acid Sequences of Tryptic Shark Na,K-ATPase Peptides. The strategy used for identification of the membrane-embedded tryptic peptides is essentially the same as that used previously for pig kidney Na,K-ATPase (Capasso et al., 1992). The 19-kDa membranes obtained after extensive tryptic digestion were subjected to size-exclusion HPLC as shown in Figure 1. The four major peptide peaks were analyzed further by polyacrylamide gel electrophoresis (see Figure 1). The β-subunit was dominant in peak 2, the 19-kDa tryptic fragment of the α-subunit was dominant in peak 3 [the partial amino acid sequence of which has been published previously (Esmann & Sottrup-Jensen, 1992)], and peak 4 contained a number of peptide bands in the 6–12-kDa range. The latter were sequenced, and the bands marked a, b, and c were found to have sequences almost identical to those of the corresponding pig kidney Na,K-ATPase fragments. In Table 1, the three sequences found are aligned with the known amino acid sequences of pig kidney (Shull et al., 1985) and *T. californica* electric organ (Kawakami et al., 1985). The sequence homology between the 19-kDa fragment and the pig kidney and *Torpedo* enzymes determined previously (Esmann & Sottrup-Jensen, 1992) is also shown for comparison. The tryptic hydrolysis sites therefore occur in positions analogous to those found with pig kidney Na,K-ATPase (Capasso et al., 1992). The N-terminal aspartic acid of band a corresponds to Asp^{68} of the pig enzyme, and likewise the N-terminal isoleucine (band b) and the N-terminal glutamine (band c) correspond to Ile^{263} and Gln^{737}, respectively, of pig kidney tryptic fragments. Amino acid sequences of other bands from gel electrophoresis of peak 4 gave no indication of further membrane-embedded segments of the α- or β-subunits.

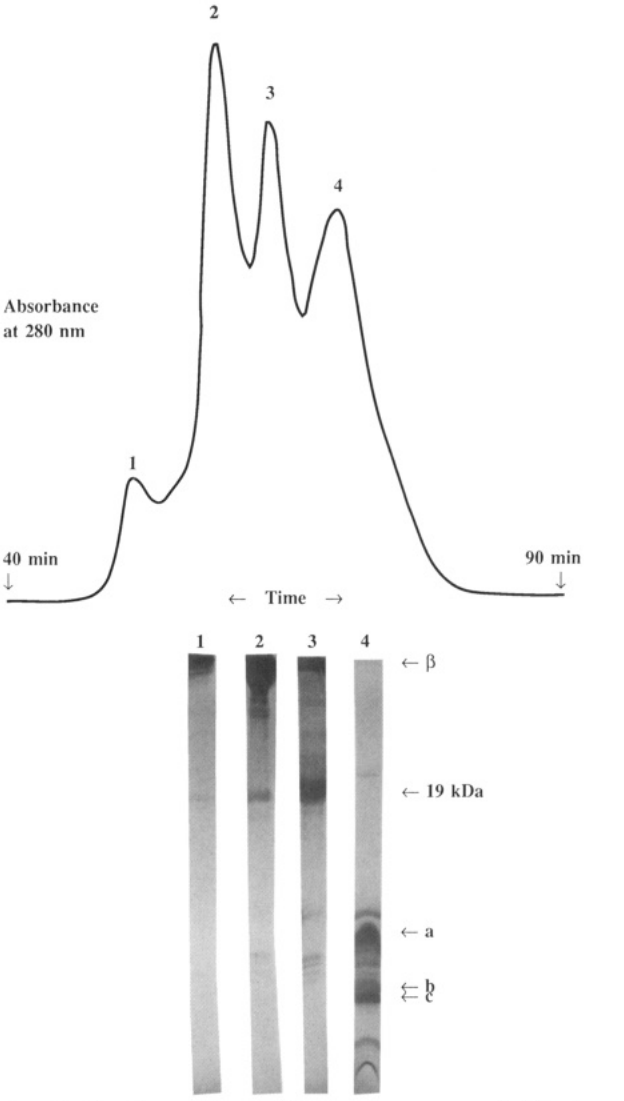

FIGURE 1: Tryptic peptides 19-kDa membranes separated by size-exclusion HPLC and resolved on 16.5% gels. Trypsinized Na,K-ATPase (1 mg) was dissolved in SDS and fractionated on TSK-G3000SW columns. The four major peaks giving the UV absorption shown were analyzed by gel electrophoresis as shown below the chromatogram. The first peak contained aggregated protein material, which was not analyzed further. Peak 2 consisted mainly of the β-subunit, and peak 3 consisted mainly of the 19-kDa peptide. Peak 4 gave a number of bands upon electrophoresis, and the bands marked a (11.5 kDa), b (8.0 kDa), and c (7.8 kDa) were found to contain parts of the amino acid sequence of the α-subunit (see Table 1). Maximum absorbance (peak 2) at 280 nm corresponds to 0.12 absorbance units.

Saturation Transfer ESR of Spin-Labeled Trypsinized α-Subunit. The molecular size and aggregational state of the Na,K-ATPase has been estimated from rotational diffusion measurements performed with the STESR method (Marsh, 1981). Sulfhydryl groups in the α-subunit were spin-labeled with a chloromercuric spin label (5-MeClHgMSL) in native

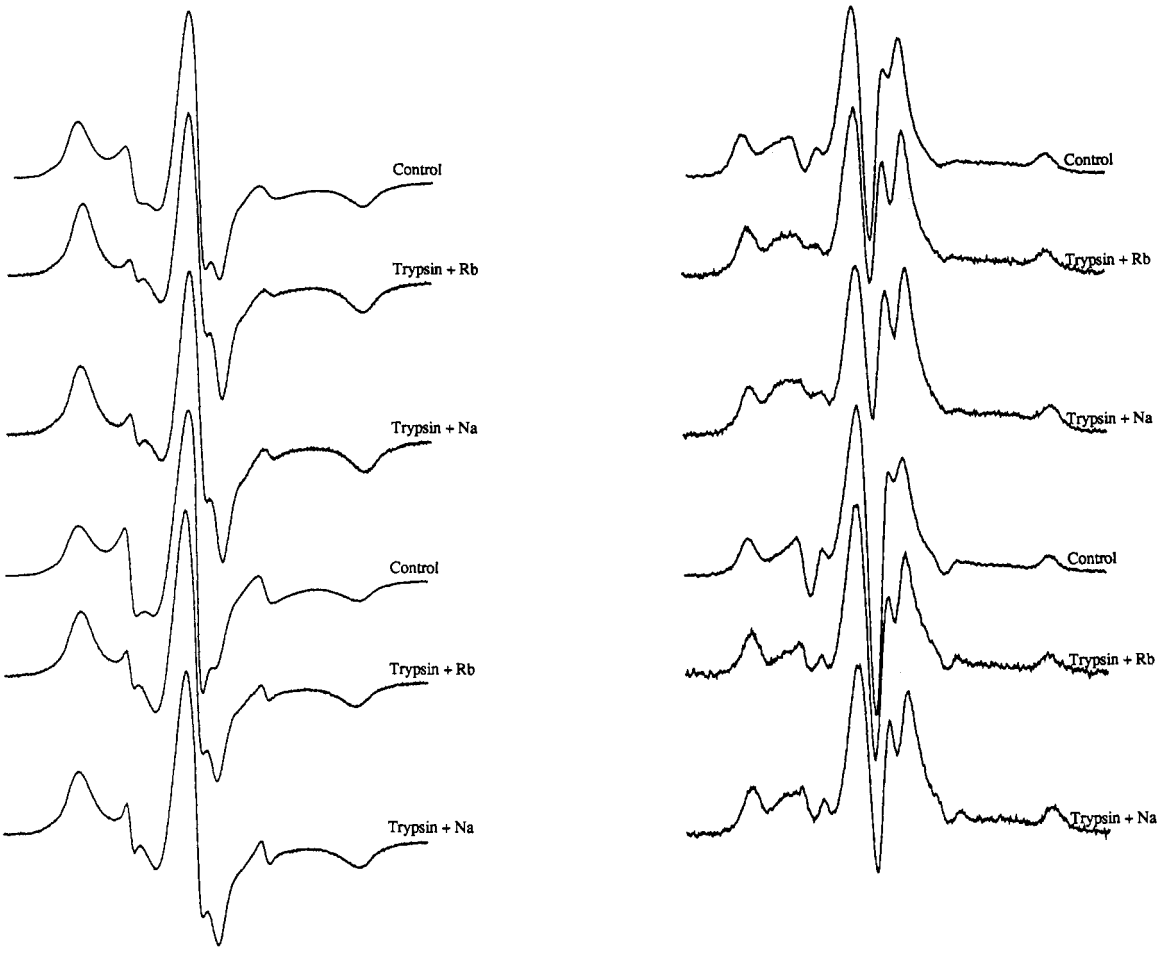

FIGURE 2: ESR spectra of Na,K-ATPase labeled with 5-MeClHgMSL. (Panel A, left) Conventional ESR spectra (V_1 display) are shown for control enzyme and for enzyme trypsinized in the presence of RbCl or NaCl, with the upper set of spectra recorded at 4 °C and the lower set at 20 °C. The membranes are suspended in 20 mM histidine (pH 7.4), 1 mM CDTA, and 100 mM RbCl (for control and Rb^+-trypsinized enzyme) or 100 mM NaCl (for enzyme trypsinized in NaCl). (Panel B, right) Second harmonic, 90° out-of-phase absorption STESR spectra (V_2'-display) of the same samples as shown in panel A. Total scan width was 100 G.

Table 1: N-Terminal Sequences of Tryptic Fragments of Shark Rectal Gland Na,K-ATPase α-Subunit[a]

Torpedo	75	DGPNALTPPPTTPWEIKF	
Shark		DGPNALTPPPTTPYDIKF	(band a)
Pig	68	DGPNALTPPPTTPWEVKF	
Torpedo	270	IATLASGLEVGQTPIAAEIEHFIHIITGVAVFLGVSFFIL	
Shark		IATLASGLDTGKTPIAKEIEHFIHIITGVAVFLGVSFFVL	(band b)
Pig	263	IATLASGLEGGQTPIAAEIEHFIHIITGVAVFLGVSFFIL	
Torpedo	743	QAADMILLDDNFASIVTGVEEGRLIFDNLKK	
Shark		QARDHILLEVNFASXVTGVXEGRLDFDNLKG	(band c)
Pig	737	QAADMILLDDNFASIVTGVEEGRLIFDNLKK	
Torpedo	840	TDKLVNERLISMAYGQIGMIQALGGFFSYFVILAENGFLP	
Shark		MDKLVNERLISIAYGQIGMIQALGGFFSYFVILAENGFLP	(19-kDa)
Pig	834	TDKLVNERLISMAYGQIGMIQALGGFFTYFVILAENGFLP	

[a] The partial amino acid sequences of bands a, b, and c from the gel electrophoresis shown in Figure 1 and of the 19-kDa fragments (Esmann & Sottrup-Jensen, 1992) are compared with the published sequences from *T. californica* (Kawakami et al., 1985) and pig kidney (Shull et al., 1985). Amino acid identities are marked with an asterisk. The first residue after the point of cleavage is indicated by the amino acid residue numbers as shown.

Na,K-ATPase membranes (Esmann et al., 1993), and it was found that about 30% of the total spin intensity remained in the 19-kDa membranes produced by trypsinization in the presence of Rb^+. 5-MeClHgMSL was chosen for spin-labeling because of the very high reactivity toward shark Na,K-ATPase and because the immobile ESR-spectra obtained for the labeled membranes are well-suited to this particular study. Spin-labeled Na,K-ATPase retained the ability to occlude Rb^+ with a slightly lower affinity for Rb^+ (not shown), and trypsinization of spin-labeled Na,K-ATPase in RbCl (see Figure 3 below) yielded digestion patterns identical to those of non-spin-labeled Na,K-ATPase (not shown). Figure 2 shows STESR spectra as well as conventional ESR spectra of spin-labeled enzyme in control membranes and in the trypsinized 19-kDa membranes. The diagnostic line height ratios and the integrated intensities obtained from the STESR spectra of Figure 2 are given in Table 2. No significant difference in these parameters is found between the control preparation and those trypsinized in Rb^+. The small proportion of mobile groups evident in the control preparation (Figure 2, panel A, uppermost spectrum) is clearly reduced upon trypsinization, but these groups do not contribute to the STESR intensity nor do they interfere with determination of the diagnostic STESR line height ratios. The corresponding effective rotational correlation times at 4 °C deduced from isotropic calibration systems are approximately 37 μs for both samples deduced from the low-field line height ratio (L″/L) and approximately 45 μs if the high-field line height ratio (H″/H) is used (Table 2). This extent of rotational mobility has been interpreted in terms of the mobility of an αβ-protomeric or $(\alpha\beta)_2$-diprotomeric state of oligomerization in

Table 2: STESR Parameters for Shark Na,K-ATPase Covalently Labeled at SH Groups with 5-MeClHgMSL[a]

sample	temp (°C)	integral (× 10^2)	L″/L	H″/H
control	4	0.23	0.77 (36)	0.55 (41)
	20	0.13	0.58 (21)	0.51 (35)
trypsinized in RbCl	4	0.24	0.80 (39)	0.59 (47)
	20	0.15	0.54 (18)	0.50 (34)
trypsinized in NaCl	4	0.42	1.13 (88)	0.74 (75)
	20	0.25	0.83 (42)	0.52 (37)

[a] The spectra shown in Figure 2 (panel B) are analyzed in the diagnostic regions of the spectra for low-field (L″/L) and high-field (H″/H) line height ratios as well as for the total integrated intensity of the STESR spectrum (I_{ST}), see Horváth and Marsh (1988) for details of analysis. The effective rotational correlation times (μs) deduced from calibrations of the line height ratios are shown in parentheses.

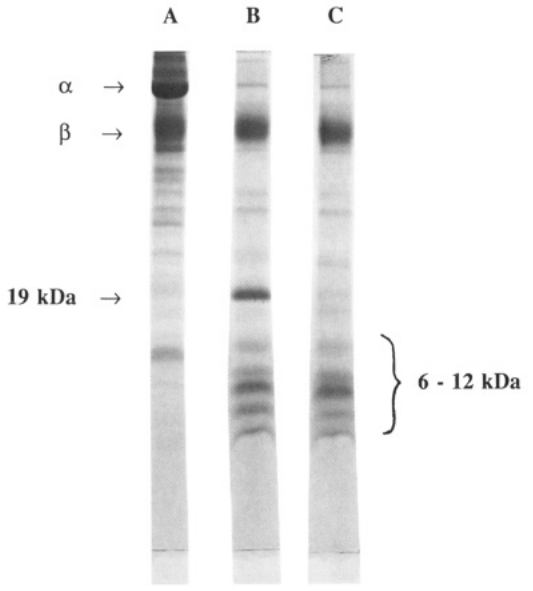

FIGURE 3: Digestion of Na,K-ATPase by trypsin in the presence of Rb^+ or Na^+. Gel electrophoretic pattern (using a 12% separation gel) of control enzyme (lane A) and enzyme trypsinized in Rb^+ (lane B) or Na^+ (lane C). The positions of the α- and β-subunits and of the 19-kDa peptide are marked.

the membrane (Esmann et al., 1987). The experiments thus indicate that the overall structure of the membrane-embedded section of the Na,K-ATPase is preserved after trypsinization, i.e., that the tryptic peptides essentially retain their native state of assembly in the membrane. No change in the overall rotation rate is to be expected (in the absence of a rearrangement of the intramembranous parts) since the extramembranous parts of the protein contribute negligibly to the overall rotational friction because they are situated in a region of very low viscosity relative to that of the membrane (Esmann et al., 1994).

Interestingly, it was found that trypsinization in the presence of NaCl instead of RbCl leads to membranes with only small tryptic fragments (6–12 kDa), i.e., further tryptic cleavage of the 19-kDa peptide occurs when Na^+ is present instead of Rb^+ (see Figure 3). These membranes were not able to occlude Rb^+. As with Rb-trypsinized membranes, about 30% of the spin intensity remained associated with the Na-trypsinized membranes. The spin-labeled and Na-trypsinized membranes have the same tryptic fragments as non-spin-labeled membranes shown in Figure 3. ESR spectroscopy of these membranes (Figure 2) indicates that the small amount of mobile spin labels is reduced upon trypsinization (panel A) and that the mobility of the spin-labeled protein determined from the STESR spectra shown in panel B is decreased relative to the control, presumably because of a rearrangement of the membrane-embedded peptides. The effective rotational correlation times at 4 °C deduced from the line height ratios (Table 2) are in the 75–90 μs range for Na^+-trypsinized membranes as opposed to 35–50 μs for both the 19-kDa membranes and the control enzyme in RbCl-containing buffer (Table 2) (control enzyme gives the same effective rotational correlation times in RbCl- and NaCl-containing buffers, not shown). On average, the effective rotational correlation time for Na-trypsinized membranes is increased by a factor of 2.1 relative to that of control membranes. The effective rotational correlation time is directly proportional to the volume of the intramembranous section of the protein [see, e.g., Esmann et al. (1994)], and the increase can thus be attributed to an approximate 2-fold aggregation, possibly accompanied also by a change in the cross-sectional asymmetry of the rotating unit.

At 20 °C the rotational mobility is increased as is expected from the lower effective viscosities in the membranes at higher temperatures. The small amount of mobile spin label attached to the protein becomes more pronounced in the STESR spectra at 20 °C, which complicates the estimation of rotational correlation times. There is no doubt, however, that the mobility is increased for all three types of membranes at 20 °C relative to 4 °C, and that Na^+-trypsinized membranes give STESR spectra that are still indicative of slower rotational motion than those of the control and 19-kDa membranes (see Table 2).

Conventional ESR of Spin-Labeled Lipids in Trypsinized Membranes. Previous ESR experiments with native Na,K-ATPase membranes containing spin-labeled lipids have revealed a motionally restricted lipid population (in addition to the fluid bilayer lipid population) that arises from the lipids in direct contact with the intramembranous perimeter of the integral membrane proteins (Esmann et al., 1985). The conventional ESR spectra of spin-labeled stearic acid, 14-SASL, in native membranes and in trypsinized membranes are compared in Figure 4 with those from membranes composed of the extracted membrane lipids. The spectral position of the motionally restricted component is indicated by an arrow in the uppermost spectrum of the figure. The spectra reveal that this component is present both in the trypsinized preparations and in control membranes. In the spectrum of the extracted lipids, which are devoid of protein, there is no indication of a motionally restricted component (lowermost spectrum in Figure 4), but rather this consists of a single component representing the fluid bulk lipid. The membranes are suspended in buffer at a pH 5.9, at which the stearic acid is protonated and displays no preferential selectivity for interaction with the protein (Esmann & Marsh, 1985). The size of the motionally restricted spin label population is therefore a direct measure of the intramembranous perimeter of the protein that is exposed to lipid. Digital subtraction of the fluid component from the upper three spectra was carried out as described previously (Esmann et al., 1985), and values for the fraction of motionally restricted lipid were found to

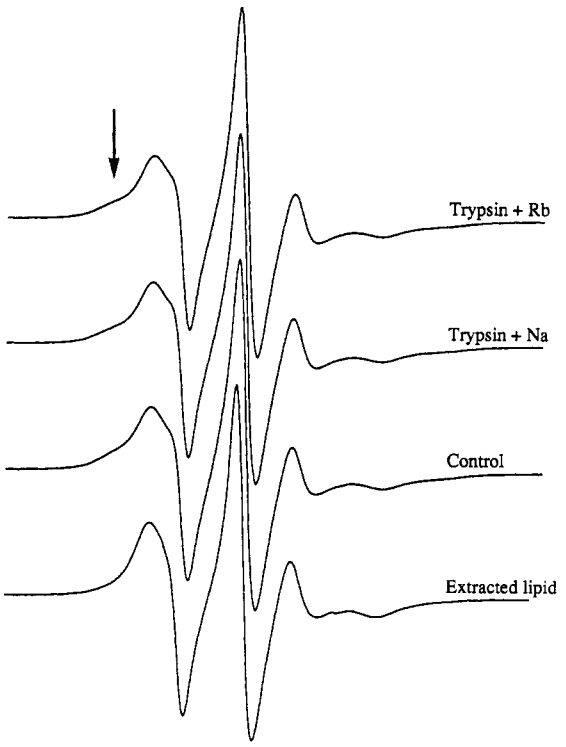

FIGURE 4: Conventional ESR spectra of spin-labeled stearic acid, 14-SASL, in Na,K-ATPase membranes. The spectra are (from top) as follows: Na,K-ATPase trypsinized in the presence of 10 mM RbCl; Na,K-ATPase trypsinized in the presence of 10 mM NaCl; native membranes; membranes formed from extracted lipids (i.e., devoid of Na,K-ATPase protein). Membranes are suspended in 10 mM Tris (pH 5.9). The temperature is 4 °C, and the total scan width is 100 G.

be in the range of 0.18–0.22, with no significant difference between control membranes and the trypsinized preparations. This constant amount of motionally restricted lipid indicates that the size of the intramembranous perimeter of the protein exposed to lipid is conserved on trypsinization.

DISCUSSION

Identity and Sequence Homology of the Membrane Domains. The identification and determination of the amino acid sequence of the membrane-embedded domains of the Na,K-ATPase from pig kidney has been performed previously, following extensive proteolytic digestion of the extramembranous portions of the enzyme (Karlish et al., 1990; Capasso et al., 1992). When proteolysis is carried out in the presence of Rb^+, the ability to occlude cations is retained and the largest membrane-associated peptide has an apparent molecular mass of 19 kDa. This latter fragment has subsequently been identified also in Na,K-ATPase membranes from *S. acanthias*, after subjection to extensive trypsinization in Rb^+, and was shown to have an amino acid sequence that is almost identical to that found for the pig kidney and *T. californica* enzymes (Esmann & Sottrup-Jensen, 1992). Here, the remaining membrane-embedded peptides of the trypsinized *S. acanthias* enzyme have been sequenced, and the sequences have been aligned with those from the other two sources (Table 1). The degree of sequence identity is only slightly less than that between the pig kidney and *T. californica* enzymes, which confirms the high degree of conservation of the membrane domains of the Na,K-ATPase. In addition, it has been found that extensive trypsinization of the *S. acanthias* enzyme in the presence of Na^+ leads to degradation of the 19-kDa membrane-embedded fragment with concomitant loss in the ability to occlude cations. This latter result establishes a direct connection between functional integrity of occlusion and intact structure of the 19-kDa fragment. It should be noted that this susceptibility to trypsin degradation in the presence of Na^+ is species dependent; the 19-kDa fragment is not degraded by trypsin in the presence of Na^+ when the kidney enzyme is studied (Capasso et al., 1992; Or et al., 1993).

Assembly of Membrane Domains after Extensive Trypsinization. The possibility to remove a large proportion of the extramembranous part of the enzyme while still retaining functionality with respect to cation occlusion allows investigation of the role of these parts of the enzyme in maintaining the structural intactness of the intramembranous domains. This has been done in the present study by determining the association state of the membrane-embedded peptides from measurements of the rotational diffusion rate of the trypsinized enzyme and of the extent of interaction with the membrane lipids. The overall rotational diffusion is determined by the cross-sectional area of the rotating species in the membrane and the extent of lipid–protein interactions by the intramembranous perimeter of the protein aggregate that is exposed to lipid (Marsh & Horváth, 1989). The lack of an appreciable difference in the STESR spectra between control enzyme and enzyme trypsinized in Rb^+ indicates that, for both systems, those of the labeled groups that give rise to an appreciable STESR intensity in the diagnostic regions (i.e., the nonmobile ones) have little or no independent motion and similar orientations relative to the protein and therefore reflect the overall protein rotation which is essentially unchanged on removing the extramembranous parts with trypsin. From this, it can be concluded that the principal features of the assembly of the intramembranous domains of the enzyme are preserved (as well as the cation-occlusion property) and that the extramembranous sections that are removed play no major role in their assembly. As for bacteriorhodopsin (Kahn & Engelman, 1992), it appears that association of the intramembranous domains is governed principally by their mutual interactions with one another and with lipid. For rhodopsin it has also been found that the two membrane-bound fragments produced by proteolysis remain closely associated both before and after bleaching (Fung & Hubbell, 1978).

In contrast to the trypsinization in the presence of Rb^+, trypsinization in the presence of Na^+ results in a reduction in mobility of the spin-labeled membrane domains, although the number of lipids interacting with the protein is not changed appreciably. This suggests that a rearrangement of the membrane domains takes place on cleaving of the extramembranous links between the transmembrane segments of the 19-kDa fragment, and this correlates with the loss of cation occlusion in these preparations. For the 19-kDa fragment, it appears that the extramembranous portions of the peptide do play a significant role in the correct assembly of the membrane-embedded segments. This is also substantiated by experiments involving a further trypsinization of the 19-kDa peptide of kidney Na,K-ATPase into 3–4-kDa fragments (Shainskaya & Karlish, 1994). It is also known that interactions between the α- and β-subunits stabilize the α-subunit against tryptic digestion (Geering et al., 1989; Noguchi et al., 1990). Further evidence for a role of the β-subunit in Rb^+ occlusion comes from experiments on the effect of degradation of the β-subunit on occlusion (Capasso et al., 1992) and from the finding that reduction of disulfide bonds in the β-subunit abolishes occlusion (Lutsenko & Kaplan, 1992).

The findings presented here open up the possibility for detailed biophysical investigations on the intramembranous sections of the enzyme by using this simplified system produced by proteolytic shaving in the presence of Rb^+.

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