# Topology of the α-Subunit of Na,K-ATPase Based on Proteolysis. Lability of the Topological Organization†

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ABSTRACT: Topology of the α-subunit of Na,K-ATPase has been analyzed utilizing proteolytic digestion. Evidence is presented for a model with 10 transmembrane segments and lability of the C-terminal domain (M7−M10). Using reconstituted proteoliposomes, inside-out oriented pumps were digested with trypsin at the cytoplasmic surface. Evidence was obtained for the M7/M8 pair and cytoplasmic splits between M8 and M9 and between M9 and M10. Because an extracellular split between M9 and M10 was also observed, using right-side-out oriented renal microsomes, we propose that the M9/M10 pair either is destabilized by cytoplasmic digestion or is intrinsically mobile. Using renal microsomes, extracellular digestion of the α-subunit by trypsin, chymotrypsin, or an endogenous protease has been observed, after incubation at 55 or at 45 °C with β-mercaptoethanol (β-ME) and *n*-butanol. Both perturbations inactivate enzyme activity. Rb ions protect against inactivation and digestion. At 45 °C, with β-ME and *n*-butanol, trypsin and chymotrypsin cut between M7 and M8 and between M9 and M10, consistent with the 10-segment model. At 55 °C, the topological organization is altered, the M8/M9 connecting loop is exposed at the extracellular surface, and an additional split between M8 and M9 is observed. Extracellular digestion of the α-subunit is associated with digestion of the β-subunit near the first extracellular S−S bridge. Rb ions protect the β-subunit. Exposure to proteases of extracellular domains of both subunits appears to be caused by disruption of subunit interactions.

Knowledge of the topological and spatial organization of transmembrane segments of a cation pump such as Na,K-ATPase[1] is necessary for an understanding of the active transport mechanism [see Glynn and Karlish (1990)]. In particular, interpretation of experiments to identify cation binding residues by chemical modification or site-directed mutagenesis depends on the presumed topological organization of the transmembrane segments [see Lingrel and Kuntzweiler (1994)].

Hydropathy plots of the catalytic subunits of the different P-type pumps are similar, suggesting that overall topological organization is similar (Jørgensen & Andersen, 1988). The four N-terminal peaks of the hydropathy plots are especially well defined. However, ambiguities in the C-terminal region have prompted development of methods to determine topology directly. As expected, each technique has advantages and limitations, and not all methods provide consistent results. One agreed result is that both N- and C-terminals are cytoplasmic [Felsenfeld & Sweadner, 1988; Antolovic et al., 1991; Modyanov et al., 1992; Thibault, 1993; Scott et al. (1992) for Na,K- and H,K-ATPase; see Karlish et al. (1993) for references to other ATPases]. Thus catalytic subunits must have even numbers of segments. The first four transmembrane segments of both Na,K- and H,K-ATPase are demonstrated clearly by proteolysis, covalent labeling, antibody binding, and *in vitro* expression experiments (Jørgensen et al., 1982; Capasso et al., 1992; Munson et al., 1991; Besançon et al., 1993; Arystarkhova et al., 1992; Bamberg & Sachs, 1994).

A previous study showed that Asn831 of the α-subunit of Na,K-ATPase is cytoplasmic [Karlish et al. (1993) numbering as in the pig α1 sequence]. Asn831 is the N-terminal residue of a 19 kDa fragment of the α-subunit produced by extensive tryptic digestion of renal Na,K-ATPase, in specific conditions, to form "19 kDa membranes" (Karlish et al., 1990; Capasso et al., 1992). The experiments utilized intact renal microsomes, which are exclusively in a right-side-out orientation (Forbush, 1982; Skriver et al., 1983), and reconstituted proteoliposomes containing pumps about 2:1 in inside-out and right-side-out orientations, respectively (Karlish et al., 1993). The experiments indicated the presence of two segments, M5 and M6, between residue 776 and 831. Studies of binding of polyclonal antibodies raised against a peptide corresponding to residues 815−827 (Ning et al., 1993) and epitopes engineered into positions 832 (Canfield & Levenson, 1993) and 828 (Yoon & Guidotti, 1994) confirm that these sections are cytoplasmic. An unresolved discrepancy is that a monoclonal antibody recognizing an epitope within the sequence of residues 810−824 binds at the extracellular surface (Mohraz et al., 1994).

As discussed in Karlish et al. (1993), the number of segments in the C-terminal 19 kDa fragment remains the

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[1] Abbreviations: β-ME, β-mercaptoethanol; DCCD, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; DOC, deoxycholate; Na,K-ATPase, (sodium plus potassium)-activated adenosine triphosphatase (EC 3.6.1.37); PKA, protein kinase A; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLCK, Nα-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone; Tricine, *N*-[2-hydroxy-1-bis(hydroxymethyl)ethyl]-glycine.

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outstanding issue. A likely number of segments is four (Karlish et al., 1993), but it could also be only two segments, as proposed by Canfield and Levenson (1993), on the basis of binding of anti-hemagglutanin antibodies to epitopes engineered into expressed pumps. Other recent work demonstrates an extracellular location of the M7/M8 connecting loop, utilizing chimeric Ca-/Na,K-pumps to show an interaction with the $\beta$-subunit (Lemas et al., 1994), or binding of antibodies (Ning et al., 1993; Mohraz et al., 1994). Nevertheless, antibody binding to intact membranes can be misleading. A requirement for detergents may reflect conformational changes of the protein rather than opening of the cells or vesicles, and certain antibodies only recognize denatured polypeptides. Other observations include identification of membrane-embedded limit tryptic fragments, corresponding to M7, M9, and M10 [see Figure 10A and Shainskaya and Karlish (1994)], or labeling of peptides corresponding to M7, M9, and M10 with hydrophobic photoactivated probes (Modyanov et al., 1992), or M9 with DCCD (Goldshleger et al., 1992). These findings suggest that the peptides could be transmembrane segments but do not prove that they actually span the membrane. Covalent labeling and tryptic digestion of gastric H,K-ATPase and digestion of sarcoplasmic reticulum Ca-ATPase, at the cytoplasmic surface, led to identification of peptides corresponding to the M1/M2, M3/M4, M5/M6, and M7/M8 pairs (Besançon et al., 1993; Shin et al., 1994). Significantly, the predicted M9/M10 pair was not observed in either system. A 4 kDa fragment of Ca-ATPase corresponding to M9 was observed (Shin et al., 1994).

In order to obtain more definitive evidence on the number of segments in the C-terminal region, Na,K-ATPase has been proteolyzed extensively at the cytoplasmic surface, using reconstituted proteoliposomes, and at the extracellular surface using intact right-side-out renal microsomes, and the membrane-embedded peptides have been identified by N-terminal sequencing. Although the $\alpha$-subunit is highly resistant to proteolysis at the extracellular surface (Forbush, 1982; Antolovic et al., 1991; Karlish et al., 1993), it has now been found that the extracellular domains become accessible to trypsin and chymotrypsin after perturbation of native structure. The results provide evidence for a model with 10 segments, and thermal lability of the C-terminal domain. Preliminary accounts of this work have appeared (Karlish, 1994; Goldshleger & Karlish, 1994).

## MATERIALS AND METHODS

*Enzyme and Vesicle Preparations.* Na,K-ATPase, with specific activities 12−17 units/mg of protein, was prepared from pig kidneys by the rapid procedure of Jørgensen (1988). Before use, the enzyme was dialyzed at 4 °C against a solution containing 25 mM histidine, pH 7.0, and 1 mM EDTA (Tris). Tryptic digestion of Na,K-ATPase to produce "19 kDa membranes" was done as in Capasso et al. (1992).

Na,K-ATPase was reconstituted into soyabean phospholipid vesicles by freeze−thaw sonication (Karlish, 1988; Karlish et al., 1993). The medium trapped within the vesicles contained 55 mM RbCl, 22 mM histidine, pH 7.0, and 0.9 mM EDTA (Tris). Proteoliposomes were centrifuged once, on columns of Sephadex G-50 equilibrated with ice-cold 60 mM choline chloride, 22 mM histidine, pH 7.0, 0.9 mM EDTA (Tris), and 10 mM RbCl [see Penefsky (1977)].

Right-side-out renal membrane vesicles were prepared by centrifuging crude microsomes on a cushion of 15% Metrizamide as described in Karlish et al. (1993). In order to remove trapped K ions, the sealed vesicles were dialyzed for 5 h at 20 °C and overnight at 4 °C against a solution containing 250 mM sucrose, 25 mM histidine, pH 7.2, and 1 mM EDTA (Tris). Choline chloride (10 mM) or 10 mM RbCl was added, and vesicles were equilibrated for several hours at 4 °C before use.

*Proteolysis.* Proteoliposomes (containing about 80 $\mu$g of protein and 3 mg of lipid per 500 $\mu$L of suspension) were incubated with TPCK-trypsin (1:7 to 1:3 w/w protease/protein) at 37 °C in the absence or presence of 1 mM $CaCl_2$. Digestion was arrested by addition of soybean trypsin inhibitor (5:1 w/w) and 0.5 mM PMSF. The proteoliposomes were diluted and washed twice by centrifugation [see Karlish et al. (1993)] in the proteoliposome suspension medium containing trypsin inhibitor and PMSF and finally were collected from the medium containing PMSF. Proteoliposome pellets equivalent to 1.5 mL of original suspension (containing 240 $\mu$g of protein) were suspended in 0.8 mL of 25 mM histidine, pH 7.0, and 1 mM EDTA, and 4 mL of chloroform/methanol (1:2 v/v) was added. After 3 days at −20 °C, precipitated protein was collected by centrifugation and dissolved in 2% SDS or the gel sample buffer.

Right-side-out renal microsomes either were digested at 55 °C or were heated to 55 °C and then cooled and digested at 20 °C or were digested at 45 °C in the presence or absence of 300 mM $\beta$-ME or 1% *n*-butanol. Digestion was done with TPCK-trypsin or with $\alpha$-chymotrypsin (1:100 to 1:5 after 55 °C heating, and 1:5 to 1:1 at 45 °C in the presence or absence of 300 mM $\beta$-ME or 1% *n*-butanol). Tryptic digestion was arrested by addition of trypsin inhibitor (5:1 w/w) plus 0.5 mM PMSF. Microsomes were washed twice at 20 °C by centrifugation (200000*g*, 1 h) in the microsome suspension medium, containing 50 $\mu$g/mL trypsin inhibitor and 0.5 mM PMSF, and were collected in a third centrifugation at 4 °C from a medium containing 0.5 mM PMSF. Chymotryptic digestion was arrested by addition of 100 $\mu$M TPCK and 0.5 mM PMSF. The microsomes were diluted into a medium containing 150 mM KCl, 2 mM EGTA, and 100 $\mu$M TPCK and 0.5 mM PMSF, washed twice in this medium at 20 °C, and finally collected by centrifugation at 4 °C. The extensive washing procedures were necessary to remove traces of trypsin or chymotrypsin adsorbed to the microsomes. The pellets were dissolved in 2% SDS, and protein was precipitated by addition of four volumes of methanol (−20 °C, overnight). Precipitated protein was collected by centrifugation and dissolved in 2% SDS prior to size-exclusion HPLC or dissolved in gel sample buffer.

*Size-Exclusion Chromatography.* HPLC was done using the TSK-G3000SW column and instrumentation described previously (Capasso et al., 1992). The elution buffer contained 100 mM sodium acetate, pH 4.5, 0.5% SDS, and 300 mM $\beta$-ME. Fractions were collected manually. Four volumes of methanol (−20 °C) were added to precipitate protein.

*Immunoprecipitation.* Protein precipitated from digested microsomes or HPLC fractions was dissolved in 1% SDS, and this solution was diluted 10-fold with a solution containing 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, and 1% Triton-X100 (TENT solution) [see Geering et al. (1985)]. Affinity purified anti-ETYY (Thibault, 1993) dissolved in 3.8 M $(NH_4)_2SO_4$ was centrifuged twice on Centricon 30 cartridges in order to concentrate the protein

(about 3-fold) and deplete the solution of $(NH_4)_2SO_4$. The IgG concentration was determined by the absorption at 280 nm. The final concentration was ≈0.6 mg/mL. Peptides dissolved in TENT solution were mixed with the concentrated IgG. The amounts in Figure 6 were 7.75 mL solution of digested peptides (~100 μg) and 2.5 mL of anti-ETYY (~1.5 mg). The mixture was shaken overnight at 4 °C. Three hundred microliters of Protein A Sepharose suspension in 20% ethanol (capacity 70 nmol of IgG) was washed three times with 2 mL of water and twice with TENT solution and was equilibrated with TENT solution for 5 h at 4 °C. The Protein A Sepharose suspension was added to the anti-ETYY peptide mixture and shaken overnight at 4 °C. The beads were washed twice with 2 mL of ice-cold TENT solution and twice with a solution containing 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 0.07% SDS. Gel sample buffer (120 μL) was added, the suspension was mixed by vortexing intermittently for 1.5 h, and the beads were removed by centrifugation leaving a solution (200 μL) of SDS-dissolved peptides and IgG. The protein was applied to four lanes of a 16.5% gel.

*SDS−PAGE, Sequencing, and Immunoblots.* Tricine-SDS−PAGE (10%, separating gel, 11.5 cm, 10−40 μg per lane, and 10% or 16.5%, 20 cm separating gel, 80−150 μg per lane) was performed as described in Schägger and von Jagow (1987) with the modifications described in Capasso et al. (1992). For sequencing, peptides were electroblotted onto PVDF paper [see Matsudaira (1987)] as described in Capasso et al. (1992). Sequencing was done on an Applied Biosystems instrument, Model 475A. Immunoblots with anti-ETYY (diluted 1:100 from a solution of 0.4 mg/mL) were performed as described in Capasso et al. (1992), after transfer of peptides to PVDF paper. Immunoblots were stained with diaminobenzidine with metal ion enhancement (Harlow & Lane, 1988).

*Functional Assays.* Na,K-ATPase activity of renal microsomes was assayed after demasking with deoxycholate (Jørgensen, 1988; Karlish et al., 1993). Rb occlusion in renal microsomes was measured as in Shani et al. (1987) after demasking with DOC, 1.5 mg/mL. The medium contained 125 mM sucrose, 12.5 mM histidine, pH 7.0, 0.5 mM EDTA, 0.5−10 mM RbCl (plus $^{86}Rb$), about 200 μg of vesicle protein treated or not treated with 2.5 mM ouabain, 1.5 mM $MgCl_2$, and 3.5 mM $P_i$ (Tris). Rb occlusion represents the ouabain-inhibited fraction of observed $^{86}Rb$ binding (about 65% at 0.5 mM RbCl).

*Materials.* Materials were of the highest grade of purity available. $^{86}Rb$ was obtained from Du Pont NEN. Protein A Sepharose 4B, P9424, soybean trypsin inhibitor, T9003, PMSF, P7626, TPCK, T4376, and diaminobenzidine D5637 were from Sigma. PVDF was from Millipore. TPCK-trypsin, 3740, 274 units/mg was from Worthington, and α-chymotrypsin, 2307, 350 units/mg was from Merck. Reagents for SDS−PAGE were of electrophoresis grade from Bio-Rad.

## RESULTS

Section A describes experiments utilizing reconstituted proteoliposomes for digestion of the α-subunit at the cytoplasmic surface. Sections B−E describe experiments utilizing right-side-out renal microsomal vesicles for digestion of the α-subunit at the extracellular surface. Results may be understood more easily by referring to the topological models in Figure 10.

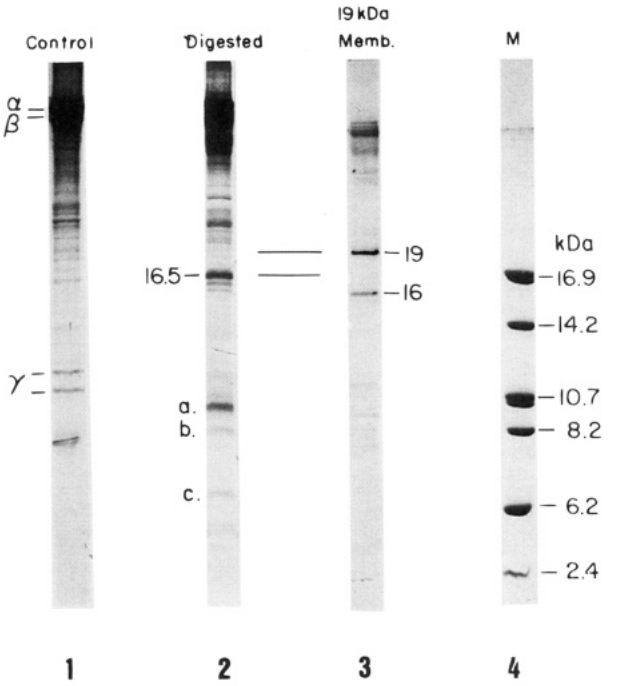


FIGURE 1: Fragments produced by extensive tryptic digestion of reconstituted proteoliposomes. Twelve milliliters of proteoliposomes (≈1.9 mg of protein) was incubated with 264 μg of TPCK-trypsin for 2 h at 37 °C in a medium containing 1 mM $CaCl_2$. All subsequent manipulations were as described in Materials and Methods. Gel was loaded with ~150 μg of protein per lane in control (lane 1) or digested (lane 2) proteoliposomes or ~30 μg of "19 kDa membranes" (lane 3) and molecular weight markers (lane 4).

*(A) Digestion at the Cytoplasmic Surface in Reconstituted Proteoliposomes.* Trypsinolysis of reconstituted proteoliposomes in a K-containing medium produces the 19 kDa C-terminal fragment by digestion of in-side-out oriented pumps, while right-side-out pumps are resistant to digestion (Karlish et al., 1993). According to a 10-segment model (Figure 10A), digestion of the 19 kDa fragment at the cytoplasmic surface could give rise to two limit fragments of 9−10 kDa, corresponding to the pairs M7/M8 and M9/M10. A model with an extracellular loop of about 70 residues (Canfield & Levenson, 1993) could lead to a limit fragment of 14−15 kDa. Previously, we reported that digestion of proteoliposomes loaded with Rb leads to accumulation of the 19 kDa fragment at 20 °C but not at 37 °C (Karlish et al., 1993). Trypsinolysis of Na,K-ATPase in the presence of Ca and Rb ions leads to destabilization of the 19 kDa fragment and its digestion to limit membrane-embedded fragments (Karlish et al., 1990; Shainskaya & Karlish, 1994). Thus, analysis of the peptides in the C-terminal region after extensive digestion of proteoliposomes at 37 °C, without or with Ca ions, has allowed us to address the questions raised above.

Proteoliposomes contain in-side-out and right-side-out orientated Na,K-pumps in a ratio of about 2:1 (Karlish et al., 1993). There are few or no functional pumps in open vesicles [see a comment by Mohraz et al. (1994)]. This conclusion is based on observations that phosphorylation, produced by addition of ATP and Na ions, is not significantly inhibited by ouabain added to the medium, and K-dependent dephosphorylation of the phosphoenzyme is negligible in the absence of valinomycin (Karlish & Stein, 1982). Thus, neither ouabain nor K ions have access to the extracellular surface, as would be the case were there unreconstituted

Table 1: Identification of Fragments Produced by Tryptic Digestion at the Cytoplasmic Surface

| apparent molecular mass (kDA) | sequence plus yield (pmol residue/cycle) | predicted fragment | calculated molecular mass (kDa) |
|---|---|---|---|
| a. 10 | L I S M A Y G Q I G<br>57 54 16 14 22 8 6 7 7 2 | L842−K931 (α) | 10.45 |
| a. 10 | G D V D P F Y Y D<br>60 37 31 14 16 11 4 3 7 | (γ) | |
| b. 8 | I A T L A S G L<br>87 33 10 5 6 2 2 2 | I263−K342 (α) | 8.44 |
| c. 6.5 | M Y P L K P T<br>88 77 38 22 10 7 3 | M973−K1012 (α) | 5.13 |

functional pumps in the preparation. Some denatured protein is present after reconstitution, but the majority is removed by centrifugation on the Sephadex columns (Rephaeli et al., 1986).

In the experiment in Figure 1, proteoliposomes were digested with trypsin for 2 h at 37 °C in the presence of 1 mM $CaCl_2$. Digestion in the absence of Ca ions produced the same fragments but at a slower rate. Protein was applied to a 16.5% Tricine gel, in order to resolve fragments in the 5−20 kDa range. In the control (lane 1) the α- and β-subunits are not resolved, but two bands typical of the γ-subunit are clearly seen. In the extensively digested sample (lane 2), about half of the α-subunit was digested, but no 19 kDa fragment appeared, as expected (see lane 3 for its expected position). Undigested α-subunit represents pumps in a right-side-out orientation (Karlish et al., 1993). The band at 16.5 kDa is a fragment of the β-subunit nicked at the extracellular surface of right-side-out oriented pumps [see Karlish et al. (1993)]. No major fragments of 14−15 kDa, predicted by the eight-segment model (Canfield & Levenson, 1993), were observed. Three clear bands (a, b, c) in the range 6.5−10 kDa appeared. Fragments a, b, and c were transferred to PVDF and sequenced (Table 1). Note the similar yields in picomoles per cycle, showing that no fragment is derived from a minor fraction of denatured protein and suggesting that all fragments were produced in 1:1 stoichiometries. Band a consists of a mixture of a fragment of α- (N-terminal Leu842, corresponding to M7/8 see Figure 10B) and a fragment of the γ-subunit (Mercer et al., 1993). Band b (N-terminal Ile263) corresponds to M3/4 in Figure 10A. Band c has N-terminal Met973, which is predicted to be located in the loop between M9 and M10 at the extracellular surface (see Figure 10A). M1/M2 and M5/M6 pairs were not observed. The poorly staining fragments just above the 10 kDa fragment were not sequenced and might correspond to these pairs, or fragments containing M1/M2 or M5/M6 might not have been efficiently precipitated. In any event, these fragments were of less interest since the objective was to identify fragments from the C-terminal region.

The 6.5 kDa fragment, N-terminal Met973, is an unexpected product of digestion of inside-out oriented pumps. Therefore, it seemed necessary to obtain additional evidence as to the fragments produced by cytoplasmic splits. An antibody to the final four residues of the α-subunit, anti-ETYY (Thibault, 1993), has provided the necessary tool. Since the C-terminal of inside-out oriented pumps should be located in the medium, trypsin should gain access to it and eventually remove it. However, removal of the C-terminal is unlikely to be the primary split. Therefore, we argued that tryptic fragments which bind the anti-ETYY, but appear only transiently, must be derived from inside-out

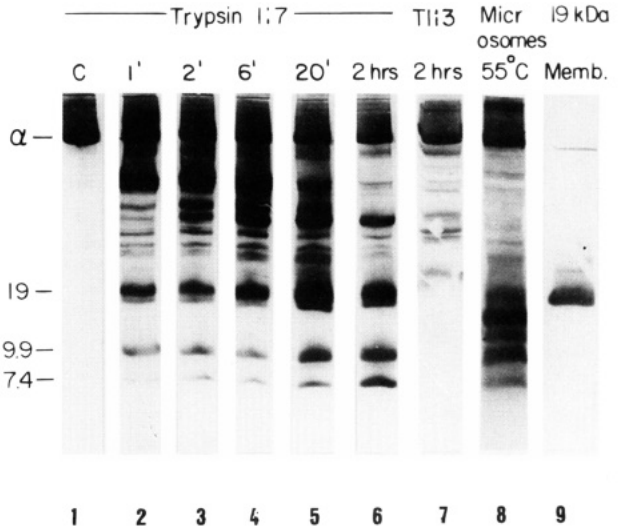


FIGURE 2: Immunoblot of fragments with intact C-terminals, produced by tryptic digestion of reconstituted proteoliposomes. Proteoliposomes were incubated at 37 °C with TPCK-trypsin (1:7 w/w) in the absence of Ca ions, for the times indicated, or with TPCK-trypsin (1:3 w/w) for 2 h. About 10 μg of protein was applied to each lane of a 10% gel. The peptides were transferred to PVDF paper, and the immunoblot was performed as described in Materials and Methods. (Lane 1) control; (lanes 2−6) 1−120 min, trypsin 1:7; (lane 7) 120 min, trypsin 1:3; (lane 8) renal microsomal protein digested with trypsin after heating to 55 °C; (lane 9) "19 kDa membranes".

oriented pumps. The immunoblot in Figure 2 shows that one can indeed detect the transient appearance and disappearance, over 2 h, of fragments with an intact C-terminal. Note in particular the 19 kDa and smaller fragments of 9.9 and 7.4 kDa. The 19 kDa fragment appears before the 9.9 and 7.4 kDa fragments (compare lanes 2−6) and begins to disappear first (compare lanes 5 and 6). The 9.9 kDa fragment, in turn, appears before the 7.4 kDa fragment (compare lanes 5 and 6, trypsin 1:7). Digestion for 2 h with a larger amount of trypsin (1:3) removes the C-terminal of most fragments smaller than the α-subunit (lane 7). The population of α-subunits resistant to the most extensive digestion bind the antibody, as expected for right-side-out oriented chains with C-terminals within the proteoliposomes (lane 7). The transiently produced fragments of 9.9 and 7.4 kDa (lanes 2−6) are inferred to be the M9/M10 pair and M10 segment, respectively, by comparison with C-terminal fragments produced by extracellular digestion of intact microsomes and identified by sequencing (see lane 8, and Table 2). The transiently produced 7.4 kDa fragment gives rise to the limit 6.5 kDa fragment in Figure 1, when the C-terminal segment is removed. The 6.5 and 7.4 kDa fragments have the same N-terminal, Met973.

*(B) Digestion at the Extracellular Surface of Renal Microsomes, after Heating at 55 °C.* In preliminary experi-

Table 2: Identification of Proteolytic Fragments Produced by Digestion at the Extracellular Surface[a]

| apparent molecular mass (kDA) | fragment | calculated molecular mass (kDa) |
|---|---|---|
| | SDS−PAGE (Figure 3) | |
| a. α 91.0 | Gly1−Arg886 | 96.8 |
| b. β 16.5 | Ala1−Arg142 | 15.3 |
| | Size-Exclusion Chromatography (Figure 5B) | |
| a. α 15.4 | Trp887−Tyr1016 | 15.5 |
| b. β 11.2 | Ala1−Lys109 | 12.6 |
| | Immunoprecipitation (Figure 6) | |
| a. α 16.2 | Arg880−Tyr1016 | 16.4 |
| | Ile888−Tyr1016 | 15.3 |
| b. α 14.9 | Glu902−Tyr1016 | 13.6 |
| c. | not identified | |
| d. α 9.8 | Gln939−Tyr1016 | 9.4 |
| e. α 9.3 | Gln939−Tyr1016 | 9.4 |
| f. β 8.6 | Ala1−Val71 | 8.2 |
| g. β 8.2 | Ala1−Val71 | 8.2 |
| h. | not identified | |
| i. α 7.1 | Arg972−Tyr1016 | 5.7 |
| j. α 6.8 | Arg972−Tyr1016 | 5.7 |

[a] Apparent molecular mass values are those estimated from electrophoretic mobility on 16.5% gels (Figures 5B and 6). These values are slightly different from those estimated from immunoblots on 10% gels. The 14.5 kDa fragment in Figure 2 (lane 2) corresponds to peptide a in Figure 5B. The 15.7, 13.1, 9.6, and 7.8 kDa peptides in Figure 2 (lane 3) appear as peptides a, b, d and e, and i and f, respectively, in Figure 6.

ments, microsomes depleted of K (see Materials and Methods) were incubated with trypsin at temperatures between 37 and 60 °C. At temperatures below 50 °C the α-subunit was resistant to digestion. At 55 °C or higher the α-subunit was digested. Subsequently, it was found that the α-subunit could be digested readily at 20 °C, after a 30 min preincubation at 55 °C, and a characteristic fragment of ≈91 kDa was produced (Figure 3, lane 2a). Essentially the same result was obtained by either protocol. The experiment in Figure 3 utilizes trypsin, but chymotrypsin is equally effective (see below). If microsomes were preloaded with RbCl, the α-subunit was completely resistant to digestion after the preincubation at 55 °C (Figure 3, lane 3). A 16.5 kDa fragment of the β-subunit (b) was observed in the latter condition (and also if digestion is done at 37 °C; Karlish et al., 1993). This fragment was not observed after incubation of K-depleted microsomes at 55 °C and may be presumed to have been digested. Thus, the presence of Rb ions during the 55 °C incubation protects both the α-subunit and the 16.5 kDa fragment of the β-subunit against trypsin.

The question arose whether the permeability barrier to trypsin is preserved during the 55 °C treatment. One indication that trypsin does not penetrate comes from the observation that proteins other than the α- and β-subunits were not digested (compare lanes 1 and 2). Previously, we have observed that, after deliberately opening vesicles with a detergent, most of the proteins are digested (Karlish et al., 1993). Confirmation that the permeability barrier was maintained at 55 °C came from sequence data. The N-terminal residues of the 91 kDa (a) and 16.5 kDa (b) fragments were found to be Gly1 of the α-subunit and Ala1 of the β-subunit, respectively (Table 2). The N-terminals of both α- and β-subunits are cytoplasmic, and both chains are digested when trypsin has access to the cytoplasmic surface, the α-subunit to many smaller fragments, and the β-subunit is clipped at Ala5 (Capasso et al., 1992; Karlish et al., 1993).

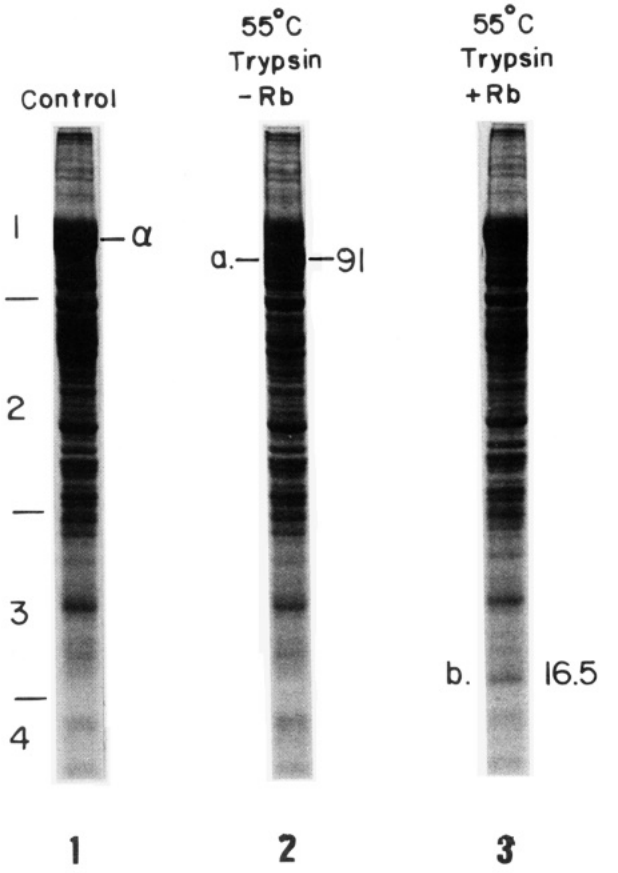


FIGURE 3: Extracellular digestion of the α-subunit in renal microsomes after heating at 55 °C. Protection by Rb ions. Rb-free or Rb-loaded renal microsomes (≈0.4 mg) were heated to 55 °C for 30 min and were digested at 20 °C with TPCK-trypsin (1:100 w/w added twice for 5 min). About 100 μg of protein was applied to each lane of a 10% gel. (Lane 1) control; (lane 2) digested, Rb-free; (lane 3) digested, Rb-loaded.

Table 3: Thermal Inactivation of Na,K-ATPase in Renal Membrane Vesicles at 55 °C: Protection by Rb Ions

| | Na,K-ATPase [μmol/(min·mg of protein)] | Rb occlusion (nmol/mg of protein) |
|---|---|---|
| control | | |
| −DOC | 0.5 | 0.37 |
| +DOC | 4.4 | |
| Rb-free (30 min, 55 °C) | | |
| −DOC | 0.44 | ≈0 |
| +DOC | 0.46 | |
| Rb-loaded (30 min, 55 °C) | | |
| −DOC | 0.54 | 0.35 |
| +DOC | 3.98 | |

Table 3 presents data on the effects of the 55 °C treatment on Na,K-ATPase activity and Rb occlusion, determined with or without opening of the vesicles with deoxycholate (DOC). In the untreated control microsomes, incubation with DOC produced a ≈9-fold increase in Na,K-ATPase activity, showing that approximately 90% of the vesicles were sealed. Heating of K-depleted microsomes at 55 °C inactivated the Na,K-ATPase activity and Rb occlusion, whereas the heating of Rb-loaded microsomes was without effect on these activities. Thus, sensitivity to digestion at the extracellular surface (K-depleted) is accompanied by functional inactivation. In addition, the unmasking effect of DOC was unchanged in the Rb-loaded microsomes, demonstrating again that the permeability barrier was preserved.

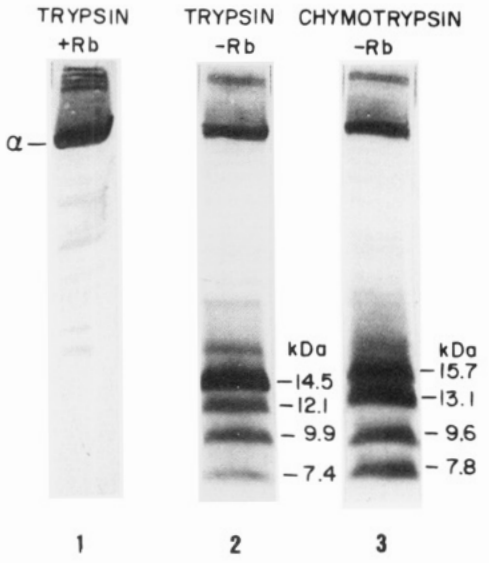


FIGURE 4: Immunoblot of fragments with intact C-terminals produced by extracellular digestion with trypsin or chymotrypsin. Microsomes were treated as in Figure 3 and digested at 20 °C for 15 min with TPCK-trypsin or α-chymotrypsin (1:50 w/w). About 10 μg of protein was applied to lanes of a 10% gel. (Lane 1) Rb-loaded, trypsin; (lane 2) Rb-free, trypsin; (lane 3) Rb-free, chymotrypsin.

The sequence data indicate that the position of the extracellular split(s) in the α-subunit must be near the C-terminal, and digestion produces fragments of 10−15 kDa. No fragments of this length were seen in the gel of Figure 3, due presumably to their small amounts. Since the C-terminal of the α-subunit is cytoplasmic, and trypsin does not appear to penetrate the vesicles, it could be expected that the small fragments would have an intact C-terminal. Therefore, the fragments should bind the anti-ETYY antibody. The experiment in Figure 4 tested this prediction. Microsomes [K(Rb)-free or Rb-loaded] were digested with trypsin or chymotrypsin, after heating at 55 °C, as in Figure 3, and then an immunoblot from a 10% Tricine gel was performed. In Rb-loaded microsomes, the α-subunit is not digested. The antibody binds to the intact α-subunit and, essentially, to no other protein in the membrane (lane 1). In K(Rb)-free microsomes, trypsin or chymotrypsin digest the α-subunit and produce four characteristic fragments which bind the antibody, with apparent molecular mass of 7.4−14.5 or 7.8−15.7 kDa, respectively (lanes 2 and 3). Residual α-subunit in lanes 2 and 3 binds the antibody, but the 91 kDa fragment does not bind the antibody, as expected. In a control experiment, in which DOC was added prior to the trypsin, the C-terminal was removed, and no fragments binding the antibody were detected (see also Figure 8 for a similar control). Thus, the experiment demonstrates that the C-terminal is indeed cytoplasmic, and the four fragments in lanes 2 and 3 represent the products of extracellular splits.

The findings in Figure 4 explain why the amounts of the 7−15 kDa fragments produced by the extracellular digestion are below the detection limit of the Coomassie stain (Figure 3). The intact α-subunit corresponds to about 10% of the membrane protein, or ≈10 μg per 100 μg applied per lane. As an upper limit, the α-subunit could give rise to 0.2−0.4 μg per lane of the four fragments, assuming that both initial digestion and recovery of the fragments were complete. In reality, lower yields could be expected.

The relative proportions of the four proteolytic fragments were not significantly changed by varying the treatments such as prior heating at 55, 60, or 65 °C, by varying the period of incubation at 55 °C above 15 min, by changing the concentration of trypsin or chymotrypsin between 1:50 and 1:5 w/w, by digesting at 20 or 37 °C, or by carrying out two cycles of heating and digestion.

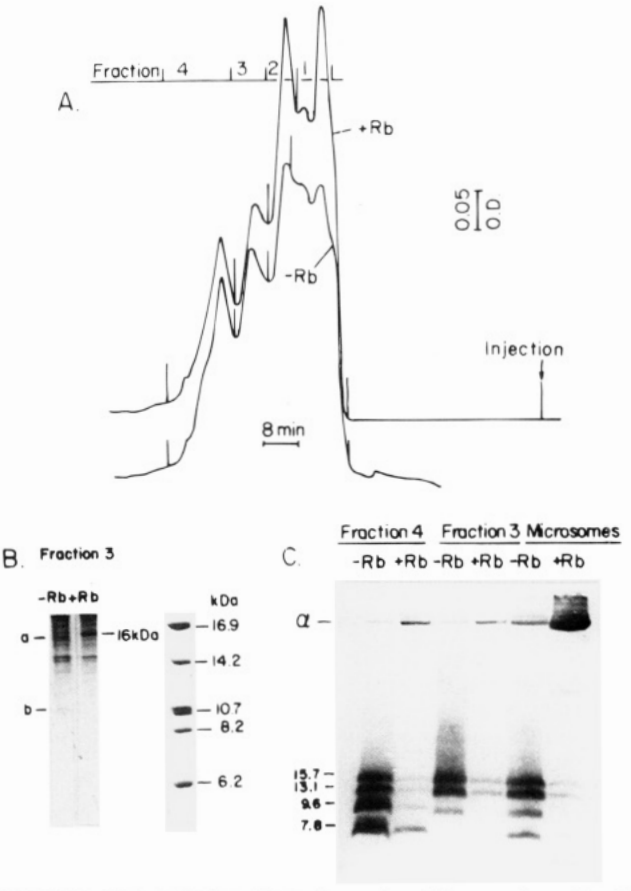


FIGURE 5: Size-exclusion chromatography of fragments produced by extracellular digestion. Rb-free (12 mg) or Rb-loaded (6 mg) microsomes were heated to 55 °C for 30 min and were digested at 20 °C, with three aliquots of TPCK-trypsin (1:20 w/w), 5 min each time. (A) 1.5 mg of precipitated protein was applied to the TSK-G3000 column, 1−5 mL fractions were collected, and methanol (4:1 v/v) was added. (B) Coomassie stain, 16.5% gel, ~120 μg per lane from fraction 3. (C) Immunoblot of fractions 3 and 4 and undigested microsomes of a separate experiment using α-chymotrypsin (1:20 w/w, three times, 5 min).

*(C) Identification of Fragments Produced by Extracellular Digestion.* Identification of the C-terminal fragments has required two stages of concentration and purification: (a) size-exclusion HPLC (Figure 5A) and (b) immunoprecipitation using the anti-ETYY antibody (Figure 6). For identification of the smaller of the four fragments, HPLC separation preceded immunoprecipitation, and, in addition, chymotrypsin was used, for the digestion produces proportionately more of the 7.8 kDa compared to the other fragments (see Figure 4).

In the experiment of Figure 5A,B, microsomes were heated to 55 °C in the absence and presence of RbCl and digested with trypsin, and after arresting digestion and washing the microsomes, the protein was precipitated, dissolved in SDS, and applied to a TSK-3000 size-exclusion HPLC column [Figure 5A; see Capasso et al. (1992)]. Four major peaks were observed. Fractions 1−4 were collected, protein was precipitated, and samples of about 100 μg of protein were applied to Tricine gels. The molecular mass ranges of proteins in fractions 1−4 is marked in the 10% gel of Figure 3. Fraction 1 contains the α-subunit and is clearly reduced

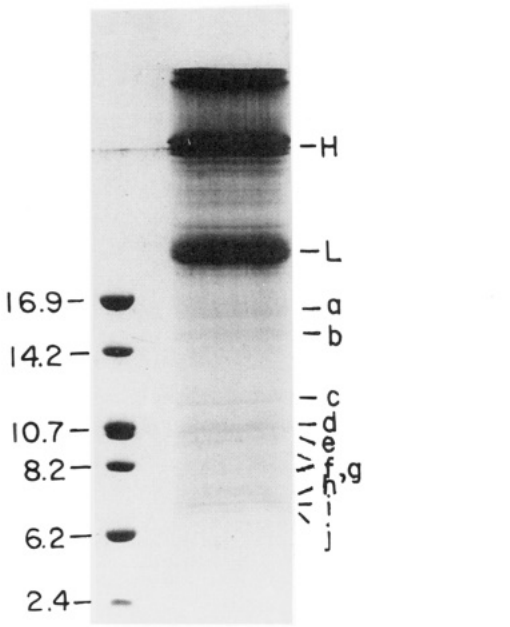


FIGURE 6: Immunoprecipitation of fragments with intact C-terminals produced by extracellular digestion. Rb-free microsomes (40 mg) were heated to 55 °C for 30 min, cooled to 20 °C, and digested with α-chymotrypsin (1:30 w/w, 20 min, added twice). After arrest of digestion and washing and precipitation of protein, SDS-solubilized protein (≈2 mg) was applied to the TSK-G3000SW column. This was repeated six times. Fractions 3 and 4 were combined and protein was precipitated. Thereafter immunoprecipitation was done exactly as described in Materials and Methods. The figure shows Coomassie stained 16.5% gel.

in the chromatographic profile of the Rb-free sample in Figure 5A. Fractions 3 and 4 were applied to a 16.5% gel, with the object of identifying bands of 7−15 kDa, appearing in the K(Rb)-free sample and absent in the Rb-loaded sample (Figure 5B). In fraction 3, two bands marked a (15.4 kDa) and b (11.2 kDa) were observed in the K(Rb)-free samples. The 16 kDa fragment of the β-subunit was much reduced in the K(Rb)-free sample. No differences were observed in the proteins of fraction 4. Fragment a was transferred to PVDF paper and was sequenced. In two experiments, the sequence XINDE was found, identifying the peptide as Trp887−Tyr1016 of the α-subunit (Table 2). Fragment b, N-terminal Ala1, is a product of extracellular tryptic digestion of the 16 kDa fragment of the β-subunit. Figure 5C shows an immunoblot of fractions 3 and 4 from an HPLC run of microsomes digested with chymotrypsin, when equal amounts of protein were applied in each lane. The 7.8−15.7 kDa fragments are concentrated 2−3-fold compared to the microsomes and separated from any undigested α-subunit.

In a preliminary immunoprecipitation experiment, the quantity of anti-ETYY antibody was titrated against a fixed amount of microsomes digested with chymotrypsin, at molar ratios of antibody to peptides from far below 1:1 to about 1:1. Comparisons of immunoblots of the digested microsomes and the precipitated peptides showed that the efficiency of immunoprecipitation is about 20%, at ∼1:1 molar ratios of antibody to peptides. The preparative experiment (Figure 6) utilized K(Rb)-free microsomes containing 40 mg of protein, comprising about 4 mg or roughly 40 nmol of α-subunit. After HPLC, as in Figure 5A, protein precipitated from combined fractions 3 and 4 was dissolved in SDS and diluted 10-fold with TENT solution, 1.5 mg or 10 nmol of the affinity purified anti-ETYY was added (estimated as ∼1:1 molar ratio of antibody to peptides), and the mixture was incubated overnight at 4 °C with shaking. After overnight incubation with Protein A-Sepharose beads (binding capacity, 70 nmol of IgG), the immunoprecipitated and SDS-solubilized peptides were applied to a 16.5% Tricine gel (Figure 6). Heavy (H) and light (L) chains of the immunoglobulin were prominent as expected. Ten bands (marked a−j) were observed in the 7−16 kDa range and were candidates as fragments cut at the extracellular surface. Bands a−j were transferred to PVDF paper and sequenced for 5−8 cycles (see Table 2). Six of the 10 bands (a, b, d, e, i, and j) were identified as chymotryptic fragments of the α-subunit, and two bands (f, g) were fragments of the β-subunit. Bands i and j (N-terminal Arg972) ran as a doublet with sequence, determined as RMXPL and RMYPLK, respectively. Bands f and g (N-terminal Ala1) derive from the β-subunit and ran as a doublet with sequences ARGPAKXE and ARGPA, respectively. Bands e and d (N-terminal Gln939) ran as a doublet with the same N-terminal sequence QQGMKNK. Band b (N-terminal Glu902) gave the sequence EQRKIVE, and band a (N-terminal Ile 888 or Arg 880) consisted of two peptides, a major sequence INDXEDS and minor sequence RVNXXD. The location of the latter in the primary sequence is adjacent to that of the tryptic peptide identified from Figure 5B. The yields of residues in all cases were between 1−7 pmol per cycle. These amounts were sufficient for reliable identification, and were of the order expected, in view of the predicted yield for all the peptides in the experimental manipulations before and during immunoprecipitation and further losses to be anticipated in gel electrophoresis, transfer to PDVF, and sequencing itself.

The data demonstrate the existence, after the 55 °C heating, of a stretch of peptide at least 90 residues long at the extracellular surface, with putative M8 and M9 located outside the membrane (see Figure 10D). Clearly, the question arose whether this is the native topological organization or whether the heating to 55 °C caused the topology to be altered.

*(D) Digestion at the Extracellular Surface at 45 °C. Effects of β-ME and n-Butanol.* One way to address the question just raised was to search for conditions allowing extracellular digestion with less perturbation of the protein. The α-subunit is not significantly cut at the extracellular surface by chymotrypsin at 45 °C or lower temperatures. Reduction of disulfide bridges in the β-subunit at 40−50 °C is associated with inactivation of Na,K-ATPase activity (Kawamura & Nagano, 1984; Kirley, 1990) and Rb occlusion (Lutsenko & Kaplan, 1993). Forte and Chow (1993) have shown that at 44 °C the homologous gastric H,K-ATPase is inactivated either by β-ME or by aliphatic alcohols, and the actions of the two kinds of agent are cooperative. Therefore, we explored the effects of β-ME and *n*-butanol on extracellular digestion at 45 °C (Figures 7 and 8 and Table 4).

Figure 7 shows a Coomassie stained gel (A) and immunoblots (B) of microsomes digested with chymotrypsin at 45 °C in the presence of 1% *n*-butanol, 300 mM β-ME, or a combination of both agents (lanes 6, 7). Evidently, the α-subunit was partially digested to the 91 kDa fragment in the presence of either *n*-butanol (lanes 2, 3) or β-ME (lanes 4, 5), and the combined effect was much more pronounced (compare the 30 min time points). Other proteins were not cut. As seen from the immunoblots, digestion was more specific than that at 55 °C, in that only the 7.8 kDa fragment and a lesser amount of the 13.1 kDa fragments were

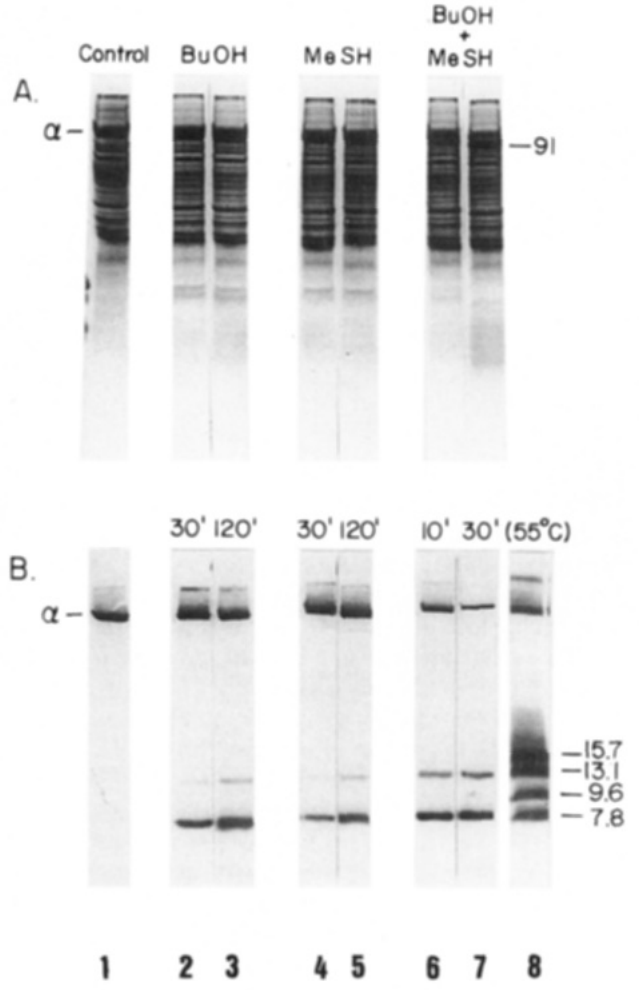


FIGURE 7: Extracellular digestion of the α-subunit at 45 °C. Effects of β-ME and *n*-butanol. Rb-free microsomes (1.6 mg of protein) were incubated at 45 °C in the presence of 300 mM β-ME or 1% *n*-butanol or both reagents, with α-chymotrypsin (1:1 w/w) for 10−120 min. (A) Coomassie stain. (B) Immunoblot. Lane 1, undigested control; Lane 2, 1% butanol, 30 min; lane 3, 1% butanol, 120 min; lane 4, 300 mM β-ME, 30 min; lane 5, 300 mM β-ME, 120 min; lane 6, 1% butanol plus 300 mM β-ME, 10 min; lane 7, 1% butanol plus 300 mM β-ME, 30 min; lane 8 (B) digestion after heating at 55 °C.

produced, while the 15.7 and 9.6 kDa fragments were completely absent (compare lanes 2−7 and 8). The control experiment in Figure 8A shows that when the vesicles were opened with deoxycholate (lanes 2, 4), the chymotrypsin removes the C-terminal, indicating that the C-terminal is cytoplasmic, and the vesicles are intact at 45 °C in the presence of 1% *n*-butanol. Figure 8B (lane 2) demonstrates complete protection by Rb against digestion in these conditions, as seen also in Figure 3 (lane 3).

Treatment of microsomes at 45 °C for 1−2 h with either β-ME or *n*-butanol is associated with partial inactivation of Na,K-ATPase activity, and the combination of β-ME with *n*-butanol completely inactivates the enzyme (Table 4). At 37 °C the combination of β-ME with *n*-butanol is also partially effective. Despite the partial inactivation, immunoblots of microsomes digested at 37 °C in the presence of β-ME with *n*-butanol show that the 7.8 kDa fragment is not produced. Thus, digestion occurs only at 45 °C, in the presence of *n*-butanol or β-ME.

*(E) Digestion at the Extracellular Surface by an Endogenous Protease.* In conditions which permit extracellular digestion of the α-subunit by exogenous proteases, the

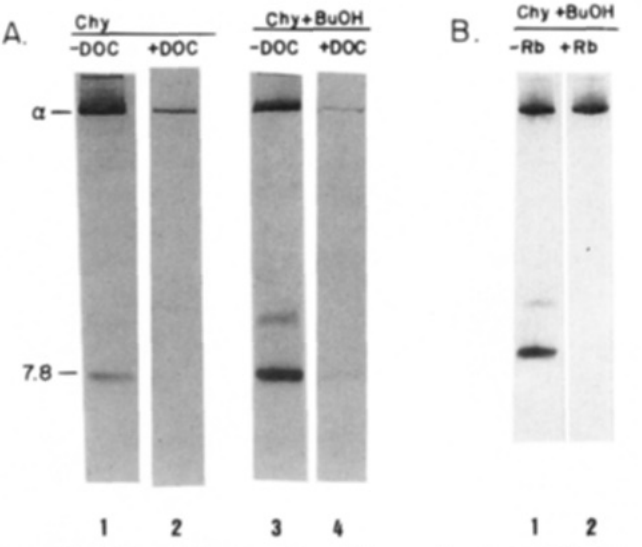


FIGURE 8: Extracellular digestion of the α-subunit at 45 °C. (A) Removal of the C-terminal in the presence of deoxycholate. (B) Protection by Rb ions. (A) Rb-free microsomes (0.75 mg) were incubated with α-chymotrypsin (1:2 w/w) for 60 min at 45 °C without or with 1% butanol, in the absence or presence of deoxycholate (1.1 mg/mL). (B) Digestion of Rb-free or Rb-loaded microsomes as in panel A. The figure shows immunoblots.

Table 4: Effects of β-ME and *n*-Butanol on Na,K-ATPase in Renal Vesicles

| condition | Na,K-ATPase [μmol/(min·mg)] | % of control |
|---|---|---|
| control | 4.4 | |
| 45 °C, 2 h | 3.54 | 80 |
| 45 °C, 2 h + β-ME | 1.3 | 30 |
| 45 °C, 1 h + butanol | 1.66 | 38 |
| 45 °C, 1 h, β-ME + butanol | 0 | 0 |
| 37 °C, 1 h, butanol + β-ME | 1.5 | 34 |

α-subunit is partially cut by an endogenous protease present in the renal microsomal vesicle (Figure 9A). The 91 kDa fragment is produced, but other proteins do not appear to be split. A striking feature is that the endogenous proteolysis is quite specific, producing only a single fragment recognizing the anti-ETYY antibody, at either 55 or 45 °C plus *n*-butanol (Figure 9B). This 16.5 kDa fragment is slightly longer than the largest fragment produced by tryptic or chymotryptic digestion. At 45 °C plus *n*-butanol, in the presence of DOC, the endogenous protease(s) digested many of the microsomal proteins, and the α-subunit was digested to numerous fragments with an intact C-terminal (lanes 4 in Figure 9A,B). The endogenous protease was not inhibited by a cocktail of iodoacetamide, EGTA, TLCK, PMSF, and TPCK. The identity of the protease is unknown, but specificity of the extracellular split of the α-subunit and lack of digestion of other proteins (Figure 9) might imply the existence of a specific association with the pump.

## DISCUSSION

Figure 10A presents a 10-segment model of the α-subunit in the native state, Figure 10B, the result of extensive digestion at the cytoplasmic surface, Figure 10C the effect of treatment at 45 °C with *n*-butanol and β-ME and extracellular splits, and Figure 10D the proposed change in topology after heating at 55 °C and extracellular splits. In a preliminary account of the extracellular digestion, the possibility of an eight-segment model was raised tentatively

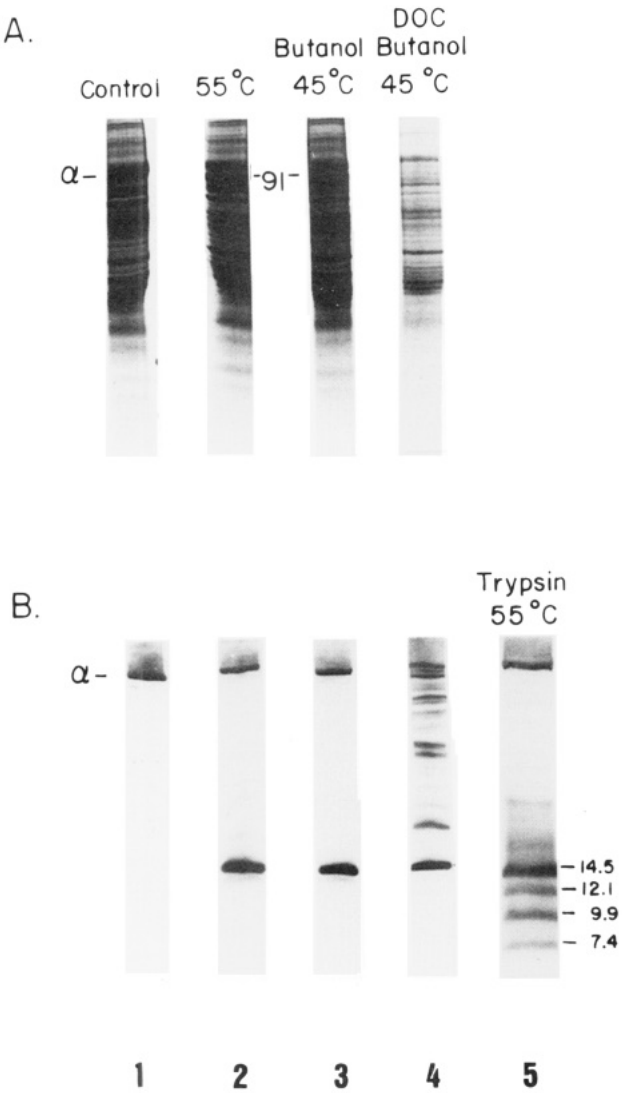


FIGURE 9: Extracellular digestion of the α-subunit by an endogenous protease. (A) Coomassie stain. (B) Immunoblot. Lane 1, control; lane 2, incubated at 55 °C; lane 3, incubated at 45 °C with 1% butanol; lane 4, as lane 3 plus DOC at 1.1 mg/mL; lane 5 (B), tryptic digestion after 55 °C heating. In each case incubation was for 30 min.

[Karlish, 1994; see Canfield and Levenson (1993)], but the present results are incompatible with that concept. Native topological organization is assumed to be retained when the pump is reconstituted into proteoliposomes, consistent with retention of its functional properties [see Karlish et al. (1993)]. We focus here on the final four segments M7−M10, since the prior evidence for M1−M6 is strong.

*(1) Cytoplasmic Digestion.* According to the 10-segment model (Figure 10A), extensive proteolysis at the cytoplasmic surface should produce two limit fragments of 10−11 and 9−10 kDa corresponding to M7/M8 and M9/M10, respectively. In reality, digestion produced two limit fragments (10 and 6.5 kDa, Figure 1) and two transiently appearing fragments (9.9 and 7.4 kDa, Figure 2) from the C-terminal domain.

The limit fragment of 10 kDa, N-terminal Leu842, has both N- and C-terminals at the cytoplasmic surface, Lys943 or Lys945 being likely C-terminal tryptic split sites. The N-terminal of the transient fragment of 9.9 kDa (Figure 2) is a tryptic site near Gln939, the N-terminal residue of the 9.8 and 9.3 kDa fragments e and d, in Table 2, probably Arg934. The result demonstrates clearly that the M8/M9 connecting loop is cytoplasmic. Thus, the 10 kDa fragment, which begins in the M6/M7 and ends in the M8/M9 connecting loop, contains the M7/M8 pair predicted by the 10-segment model. The existence of M7/M8 is confirmed by evidence for an M7 segment based on extracellular digestion (see below). The 10 kDa peptide is similar to fragments (10−11 kDa) observed by digestion of both H,K-ATPase and Ca-ATPase at the cytoplasmic surface (Besançon et al., 1993; Shin & Sachs, 1994). The present findings are consistent with the other evidence for an extracellular location of the M7/M8 connecting loop on Na/K-ATPase (Lemas et al., 1994; Ning et al., 1993; Mohraz et al., 1994) and that from binding of antibodies to Ca-ATPase and H,K-ATPase (Clarke et al., 1990; Mathews et al., 1990; Mercier et al., 1993).

Additional recent evidence that the M8/M9 loop of Na,K-ATPase is cytoplasmic comes from covalent labeling with pyridoxal phosphate (J. Kyte, personal communication) and phosphorylation by protein kinase A of Ser936 in intact cells (Beguin et al., 1994). On the basis of epitope tagging, an extracellular location of the M8/M9 loop was suggested (Canfield & Levenson, 1993), but a recent study indicates a cytoplasmic location (Yoon & Guidotti, 1994). A cytoplasmic location of this loop, KTRRNSVFQQGMKNK, is predicted from the excess of positive charge (Von Heijne, 1992).

The limit fragment 6.5 kDa, N-terminal Met973 corresponds to M10 alone, without the C-terminal (Figure 1). The transiently appearing fragments with intact C-terminals, 9.9 and 7.4 kDa, correspond to the M9/M10 pair and M10, respectively (Figure 2). The observation of a split between M9 and M10 at the cytoplasmic surface in the proteoliposomes (Figures 1, 2) but at the extracellular surface in microsomes (Figure 7) appears paradoxical. It raises a question whether M9/M10 are true transmembrane segments or whether the M9/M10 domain is anchored in the membrane without spanning it. Determination of topological organization of a glutamate receptor (Hollman et al., 1994) has provided a recent example of a putative transmembrane segment which turns out to be membrane-associated without traversing the membrane. However, were M9/M10 only anchored in the membrane at the cytoplasmic surface, an unlikely explanation of the selective extracellular split between M9 and M10 in microsomes would be required (Figure 7). Treatment at 45 °C with *n*-butanol or *β*-ME would have to displace the membrane-anchored segments toward the exterior, not randomly but specifically, allowing access of chymotrypsin to only one (Leu971−Arg972) of many potential chymotrypsin-sensitive bonds in this region.

One explanation of the experimental paradox that the M9/M10 connecting loop can be cut at either surface is that M9 and M10 are transmembrane segments in the native state, but the initial cytoplasmic splits destabilize the M9 and M10 segments, due to disruption of protein−protein interactions. It appears from Figure 2 that the M6/M7, M8/M9, and M9/M10 connecting loops and then the C-terminal itself are digested in that order, implying that earlier tryptic splits facilitate later splits. For example, digestion between M8 and M9 may destabilize the M9/M10 pair, causing the Arg−Met bond in the sequence VALRMYPL to be split at the cytoplasmic surface. The cytoplasmic split between M9 and M10 in Na,K-ATPase (Figure 2) is similar to observations on digested gastric H,K-ATPase (Besançon et al., 1993) and sarcoplasmic reticulum Ca-ATPase (Shin et al., 1994),

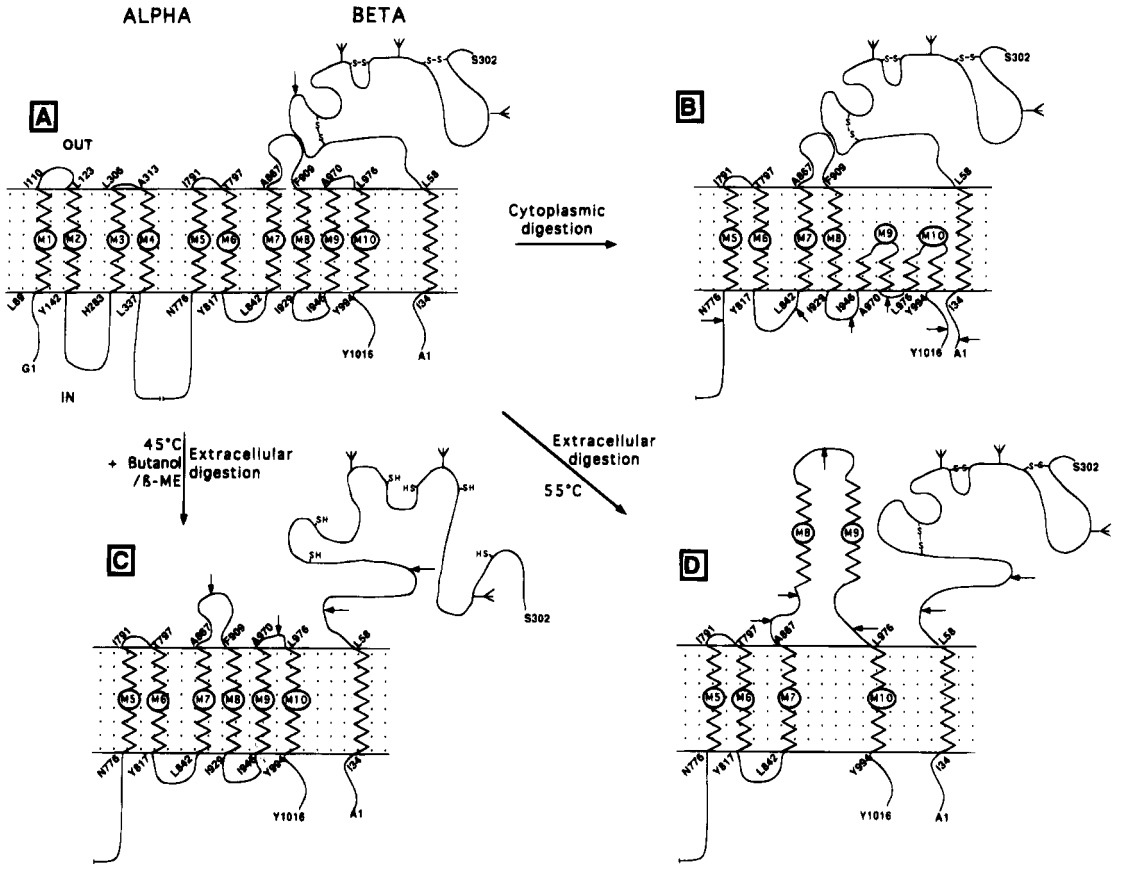


FIGURE 10: Topological models. Arrows indicate the approximate location of proteolytic splits.

suggesting that the same factors affect the stability of M9/M10 in all three pumps. Because Ca-ATPase does not have a $\beta$-subunit, extra-membrane protein-protein interactions may occur within $\alpha$-subunits.

An alternative explanation of the experimental paradox is that the M9/M10 transmembrane pair is intrinsically mobile, so that the connecting loop is accessible to proteases at either surface. The order of splits of fragments at the cytoplasmic surface (Figure 2) is explained less easily by this model but does not exclude it.

Evidence from other P-type pumps includes the *in vitro* expression assay which shows that sequences containing M9 and M10 of H,K-ATPase are capable of spanning the membrane (Bamberg & Sachs, 1994). Experiments with fusion proteins support a 10-segment model for a bacterial P-type Mg-ATPase and an extracellular location of the M9/M10 connecting loop (Smith et al., 1993).

Considering all of the evidence, one can conclude that the M9/M10 pair are normally transmembrane segments, which are either destabilized by digestion at the cytoplasmic surface or perhaps are intrinsically mobile.

*(2) Extracellular Digestion.* After heating at 55 °C, or at 45 °C in the presence of either or both $\beta$-ME or *n*-butanol, trypsin or chymotrypsin cut the $\alpha$-subunit at the extracellular surface. Both N- and C-terminal residues are protected against digestion, as shown by sequencing of the 91 kDa fragment and binding of anti-ETYY to the smaller proteolytic fragments. These observations confirm that N- and C-terminals are located at the cytoplasmic surface.

Digestion produced a 91 kDa fragment beginning at the N-terminal Gly1 and ending near Trp887, the N-terminal of the 15.4 kDa C-terminal tryptic fragment (Figure 5B and Table 2). It is unlikely that native topology of the 91 kDa fragment was altered by the heating. Had any transmembrane segments become exposed to the exterior, one could not expect the fragment to remain undigested, for there are numerous potential chymotryptic cleavage sites in all transmembrane segments. Since previous work showed that Asn831 is cytoplasmic (Karlish et al., 1993), one may deduce the existence of a transmembrane segment between Asn831 and Trp887 (M7 in Figure 10A, Leu842–Ala867).

Chymotryptic or tryptic digestion, after heating at 55 °C, produced the four characteristic fragments with N-terminal residues identified as (1) Arg880 and Ile888, or Trp887 (trypsin), (2) Glu902, (3) Gln939, and (4) Arg972 (Table 2; see Figure 10D). Thus, at least 92 residues were exposed to the proteases at the extracellular surface. On the basis of Figure 10A, the splits at Ile888 and Glu902 (between M7 and M8) and at Arg972 (between M9 and M10) were predictable, but that at Gln939 was unexpected because it lies between M8 and M9 and should be cytoplasmic. It seemed questionable whether a long extracellular loop is a native topological feature, for it includes two hydrophobic sequences, M8 and M9 in Figure 10A. Two pieces of evidence indicate that the 55 °C heating exposed the M8/M9 connecting loop at the extracellular surface (Figure 10D). First, if the long extracellular loop in Figure 10D represented a native structure, a 17–18 kDa limit fragment should be produced by extensive digestion at the cytoplasmic surface. However, 17–18 kDa fragments were not observed in the proteoliposome experiments (see Figure 1, lane 2). Second, chymotryptic digestion at 45 °C in the presence of either or both $\beta$-ME and *n*-butanol did not cut the $\alpha$-subunit at Gln939 between M8 and M9. The result in Figure 7 could be expected for a milder perturbation which allows access of the protease to the extracellular loops between M7/M8 and

M9/M10, without changing the position of transmembrane segments (Figure 10C). A recent report that heating at 55 °C causes exposure of the PKA phosphorylation site to the exterior provides additional evidence for externalization of the M8/M9 connecting loop (Arystarkhova et al., 1995).

In Figure 10D the loop of 105 residues including M8 and M9 is depicted as being totally ejected from the membrane in order to emphasize the change in topology. In reality, it is unlikely that M8 and M9 are physically outside the lipid. Also, since increasing the amounts of proteases or time of incubation did not digest all of the exposed portion to the smallest fragment (7.4 kDa), there might be an ensemble of states with different degrees of exposure of the M8/M9 loop.

*Thermal Destabilization of Native Topology. Implications for α−β Interactions.* Correct interactions between α- and β-subunits are essential for assembly and targeting to the cell membrane and for stabilization of functional pumps (Geering, 1991). The following observations suggest that disruption of the subunit interactions at the extracellular surface exposes the M9/M10 and M7/M8 loops to proteases, at 45 °C with β-ME and *n*-butanol (Figure 10C), while additional perturbations at 55 °C expose the M8/M9 connecting loop (Figure 10D). Presumably, either perturbation disrupts cation occlusion sites and ATPase activity. First, topological lability is restricted to the C-terminal domain of the α-subunit. There is now good evidence that the loop between M7 and M8 of both Na,K-ATPase and H,K-ATPase interacts strongly with the extracellular domain of the β-subunit (Lemas et al., 1994; Shin & Sachs, 1994). Second, the extracellular domains of the 16 kDa fragment of the β-subunit also becomes accessible to proteases, and Rb ions protect both subunits (Figures 3 and 8). Third, the effect of β-ME (Figure 7) is suggestive of reduction of S−S bridges in the β-subunit (Kawamura & Nagano, 1984; Kirley, 1990).

Interactions between the α- and β-subunits are known to affect ATPase activity and cation binding (Kirley, 1990; Eakle et al., 1994; Jaunin et al., 1993; Lutsenko & Kaplan, 1993). Kirley (1990) proposed that reduction of S−S bonds and possible solvent-like effects of β-ME disrupt α−β interactions and induced a delocalized denaturation of the α-subunit. The results in Figure 7 and Table 4 [and those of Forte and Chow (1993) on H,K-ATPase] support this idea, for they appear to indicate cooperative effects of reduction of S−S bridges and weakening of hydrophobic interactions by the aliphatic alcohol [see also Jaunin et al. (1993)]. By contrast, Lutsenko and Kaplan (1993) proposed that the β-subunit is directly involved in the gating mechanism of cation occlusion, but the experiments on inactivation of Rb occlusion in "19 kDa membranes" by DTT can be interpreted equally well as indicating an indirect role of the β-subunit, e.g., maintenance of stability.

Segments(s) of the β-subunit interacting with the α-subunit have not yet been unambiguously identified. Observations with chimeric proteins suggest that the N-terminal tail and transmembrane domain of the β-subunit is important for assembly, while both the transmembrane segment and extracellular domain may be important for functional interactions (Jaunin et al., 1993; Eakle et al., 1994). Other domains in the C-terminal of the β-subunit are involved, but these could be required for correct folding of this subunit (Beggah et al., 1993). It has been suggested that the segment between the first S−S bridge (Cys125−Cys148) interacts with the α chain (Kawamura et al., 1994; Lemas et al., 1994). The present finding that proteolysis after heating removes residues 71−143 of the β-subunit is compatible with the latter proposal (see Figure 10C,D). A previous observation, using "19 kDa membranes", that the 16 kDa fragment (Ala5−Arg142) of the β-subunit was digested to a fragment of 9.7 kDa (Thr27−Lys112), in the absence of occluded Rb ions, also suggested that the sequences removed, Asp113−Arg142 at the extracellular surface and Ala5−Arg26 at the cytoplasmic surface, could interact with the α-subunit and affect Rb occlusion (Shainskaya & Karlish, 1994). Coimmunoprecipitation from a solution containing SDS of the digested fragment of β- (Ala1−Val71, Table 2) together with the C-terminal fragments of the α-subunit (see Figure 5B, and Table 2) suggests that there must also be strong interactions between the transmembrane domains or cytoplasmic domains of the chains. Evidently more detailed analysis and means of distinguishing direct or indirect interactions of α- and β-subunit domains is required.

*Stabilizing Effects of Rb Ions.* Thermal inactivation of purified Na,K-ATPase, with protection by K ions, was described previously (Jørgensen & Andersen, 1986). Digested "19 kDa membranes" are much more sensitive than native enzyme to thermal inactivation, and occluded cations protect strongly (Or et al., 1993; Shainskaya & Karlish, 1994). Thus, removal of the cytoplasmic loops destabilized the occlusion domain within transmembrane segments. The present observations indicate that occlusion of K(Rb) ions induces substantial stabilizing interactions in the C-terminal region (Figures 3 and 8 and Table 3). Proteolysis experiments also provide evidence for substantial structural changes in both subunits induced by occluded cations (Shainskaya & Karlish, 1994) and other pump ligands (Lutsenko & Kaplan, 1994).

The reader may wonder whether the thermally destabilized and Rb-protected C-terminal domain acts as a mobile part of the pump in its normal function. Despite the piquancy of this speculation, it is more likely that events observed in denatured or digested protein reflect the forces stabilizing transmembrane segments. Assembly of polytopic membrane proteins, particularly of bacterial origin, is thought to involve association of membrane-spanning α-helices via specific side chain interactions (Lemmon & Engelman, 1994). Stabilizing interactions of extramembrane domains may be significant only in proteins with long extramembrane loops and multisubunit structure, unlike bacteriorhodopsin, etc. In the Na,K-ATPase and H,K-ATPase, M1−M4 could be stabilized in pairs by the conventional helix−helix interactions within the membrane. In the presence of K(Rb) ions, external protein−protein interactions may stabilize M7−M10 of Na,K-ATPase and H,K-ATPase [see Besançon et al. (1993) and Shin and Sachs (1994)]. For H,K-ATPase, *in vitro* translation experiments suggest that the same conclusion could apply also for the M5/M6 pair (Bamberg & Sachs, 1994). In the absence of K(Rb) ions the proposed extramembrane interactions are weakened and proteases cut the extramembrane domains. The resulting limit fragments corresponding to M5−M10 could be distorted α-helices, unstable within the lipid, due to the presence of proline residues (M5, M9, and M10), relatively hydrophilic character (M10 of Na,K-ATPase or M7 of H,K-ATPase) or short lengths (M5/M6 and M10). Hydrophobic fragments corresponding to M8 and M10 were reported to be released from the membrane in water-soluble form (Ovchinnikov et al., 1987). The digested M10 segment might be partially solubilized by the aqueous medium because it is short and is nonhydrophobic.

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