Extensive Digestion of Na\textsuperscript{+},K\textsuperscript{+}-ATPase by Specific and Nonspecific Proteases with Preservation of Cation Occlusion Sites* 

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This paper extends our recent report that renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase is digested by trypsin in the absence of Ca\textsuperscript{2+} and presence of Rb\textsuperscript{+} ions to a stable 19-kDa fragment and smaller membrane-embedded fragments of the α chain and essentially intact β chain. These are referred to as "19-kDa membranes." Occlusion of both Rb\textsuperscript{+} (K\textsuperscript{+}) or Na\textsuperscript{+} ions is preserved, but ATP-dependent functions are lost (Karlish, S. J. D., Goldshleger, R., and Stein, W. D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4566-4570). We now show that extensive digestion with nonspecific fungal proteases (Protrase and proteinase K) alone, in combination, or after trypsin digestion can remove up to 70% of membrane protein without destroying Rb\textsuperscript{+} occlusion. In the most heavily digested membranes, the 19-kDa fragment or a slightly shorter 18.5-kDa fragment and smaller fragments of the α chain remain, whereas the β chain is largely digested, leaving smaller membrane-embedded fragments (13-15 kDa). For either trypsin or Pronase digestion, preservation of Rb\textsuperscript{+} occlusion and the specific fragmentation pattern is observed only in the absence of divalent metal ions (Mg\textsuperscript{2+} or Ca\textsuperscript{2+}) and presence of either Rb\textsuperscript{+} or Na\textsuperscript{+} or congeners ions. Tryptic digestion at pH 7.0 can split the β chain into two fragments of ≈50 and 16 kDa, with an S-S bridge. The 16-kDa fragment is protected against further digestion by the presence of Rb\textsuperscript{+} ions, but probably is not directly involved in occluding cations.

Tryptic 19-kDa membranes show a clear and reproducible fragmentation pattern in which all predicted membrane segments are identifiable. Families of fragments from 19-kDa membranes, including seven peptides of 7.6-11.7 kDa, have been separated by size-exclusion high performance liquid chromatography, concentrated, and resolved on 16.5% Tricine gels. N-terminal sequences of the different fragments have been determined after transfer to polyvinylidene difluoride paper. The most interesting findings are as follows. (a) Whereas the 19-kDa tryptic fragment begins at Asn\textsuperscript{891} as reported previously, the 18.5-kDa Pronase fragment begins at Thr\textsuperscript{834}. (b) Fragments in tryptic 19-kDa membranes of 7.6-11.7 kDa begin at Asp\textsuperscript{86}, Ile\textsuperscript{285}, and Glu\textsuperscript{737}, respectively. These include all putative transmembrane segments other than those in the 19-kDa fragment. (c) A Pronase fragment of 7.8 kDa begins at Thr\textsuperscript{834}, i.e., apparently the 19-kDa fragment has been partially cut, without loss of Rb\textsuperscript{+} occlusion. (d) Tryptic 16- and 50-kDa fragments of the β chain begin at Ala\textsuperscript{5} and Gly\textsuperscript{14}, respectively.

Immunoblots with an antibody against the terminal 4 residues of the α chain show that both the 19-kDa tryptic and 18.5-kDa Pronase fragments extend to the C terminus of the chain.

The experiments with trypsin, Pronase, and proteinase K strongly suggest that Rb\textsuperscript{+} occlusion sites are located within transmembrane segments, including those of the 19-kDa fragment and at least one of the smaller fragments. The resistance of the 19-kDa fragment to further digestion implies that it is largely embedded within the membrane and is inaccessible to essentially any protease. Different possibilities for transmembrane folding of the tryptic fragments are discussed with the purpose of constructing models of cation sites. The findings with the nonspecific proteases are most easily understood on the basis of models with 10 transmembrane segments, as proposed for sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase.

Understanding energy transduction by cation pumps requires knowledge of the structure of the cation sites, the ATP sites, and their interactions. The structure of the cation sites of the different pumps is a central and perhaps the most intriguing problem since herein lies the question of cation selectivity. In the absence of high resolution structure, possible approaches to the problem include studies of chemical modification by carboxyl-selective reagents or reactive cation analogues combined with selective proteolytic digestion, site-directed mutagenesis, observations on charge transfer properties or voltage sensitivity of pumps, and also the use of cation analogues as probes of sites in fluorescence or NMR studies (see Glynn and Karlish (1990) for a recent review).

The approach in this laboratory has been to combine selective trypptic digestion and chemical modification of renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase using the carboxyl reagent dicyclohexylcarbodiimide (DCCD).\textsuperscript{1} In experiments designed to modify the sites, a direct measure of cation binding turns out to be crucial. For this purpose, a simple but accurate assay of Rb\textsuperscript{+} (K\textsuperscript{+}) or Na\textsuperscript{+} occlusion was developed (Shani et al., 1987; Shani-Sekler et al., 1988). We reported recently that renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase can be digested extensively by trypsin, in the presence of Rb\textsuperscript{+} and absence of Ca\textsuperscript{2+} ions, to produce membranes containing

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1 The abbreviations used are: DCCD, dicyclohexylcarbodiimide; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TPCCK, tosylphenylalanyl chloromethyl ketone; TBS, Tris-buffered saline; HPLC, high performance liquid chromatography; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
a stable 19-kDa fragment and smaller fragments of the α chain and a largely intact β chain; and occlusion of both Rb⁺ and Na⁺ ions is preserved. ATP-dependent functions are lost (Karlish et al., 1990). The 19-kDa fragment begins at Asn⁶⁰¹ of the α chain and extends toward the C terminus. It was concluded that cation sites lie within transmembrane segments and that cation and ATP sites are separate; and thus, their interactions must be indirect. Subsequent studies have shown that the 19-kDa fragment and a smaller tryptic fragment are labeled specifically by dicyclohexylcarbodiimide and, in parallel, that Rb⁺ occlusion is inactivated (Goldshleger et al., 1990; Karlish et al., 1991). The selective tryptic digestion that preserves cation occlusion is important not only for the insights it provides, but also as a tool for the labeling work since nonspecific labeling is much reduced in the truncated enzyme.

A question remains whether the tryptic peptides in 19-kDa membranes are the simplest structures compatible with preservation of occlusion since any subsequent attempt to build a model of the cation occlusion site would depend on a knowledge of the essential peptide components. There are two current hypotheses. First, that the Rb⁺ binding site is highly exposed to trypsin and may or may not be involved in cation occlusion or could perhaps protect the 19-kDa fragment against further digestion. This question has been addressed by treating the Na⁺,K⁺-ATPase with the active but nonspecific fungal proteases Pronase (a mixture of exo- and endopeptidases from Streptomyces griseus) and protease K from Tritirachium album. Second, the peptide composition of 19-kDa membranes has not been fully defined, particularly peptides of 10 kDa or smaller. Reliable methods for the separation and sequencing of the small peptides have now been developed. The experiments described here show that digestion with the nonspecific proteases provides interesting information, whereas the fragmentation pattern in tryptic 19-kDa membranes is the best defined.

**EXPERIMENTAL PROCEDURES**

Na⁺,K⁺-ATPase was prepared from fresh pig kidney red outer medulla by the rapid procedure described by Järsgen (1974). Protein was determined by the method of Lowry et al. (1951), and ATPase activity was determined as described by Järsgen (1974). Specific activities were in the range of 13–20 units/mg of protein. Before use, the enzyme was dialyzed at 4 °C against 1000 volumes of a solution containing 25 mM histidine, pH 7.0, and 1 mM EDTA/Tris.

**Rb⁺ Occlusion Assays**—These were performed by a manual method (Shani et al., 1987). The standard medium contained, in a volume of 50 µl, 100 mM choline chloride, 5 mM RbCl plus molarity × 10⁻⁶ cpm Rb⁺, 1.25 mM histidine, pH 7.0, 0.5 mM EDTA, and 25–50 µg of Na⁺,K⁺-ATPase or proteolyzed membranes.

**Treatment with Trypsin, Pronase, or Protease K**—Standard conditions for tryptic digestion (unless indicated otherwise) were as follows. Na⁺,K⁺-ATPase (1.5 mg/ml) was suspended in medium containing 12 mM histidine, pH 7.0, 1.5 mM EDTA/Tris, and 10 mM RbCl and incubated at 37 °C for 1 h with TPCK-trypsin (1:10, w/w with respect to the Na⁺,K⁺-ATPase). Soybean trypsin inhibitor was added at 1:1 (w/w) with respect to trypsin; the suspension was diluted to −25 ml with a solution of 25 mM histidine, pH 7.5, 1 mM EDTA, and 2 mM RbCl, and warmed at 37 °C for 10 min; and the membranes were collected by centrifugation at 250,000 × g for 1 h. The membranes were hand-homogenized in the latter solution; and the procedure of dilution, warming, and centrifugation was repeated twice. The washing procedure removes traces of trypsin adsorbed to the membranes.

Standard conditions for digestion with Pronase or protease K were as follows. In medium containing 25 mM histidine, pH 7.5, 1 mM EDTA, and 5 mM RbCl, control enzyme or 19-kDa membranes were incubated at 37 °C for 1 h with Pronase (1:10 or 1:3, w/w, respectively), or control enzyme or Pronase-digested enzyme was incubated at 37 °C for 1 h with protease K (1:20, w/w). In each case, the suspension was diluted with ice-cold histidine/EDTA/RbCl solution to which 0.5 mM phenylmethylsulfonyl fluoride was added and centrifuged as described above, and the procedure was repeated twice.

The trypsin 19-kDa membranes or Pronase- and protease K-digested membranes were suspended at 0 °C at 1–2 mg/ml in a solution of 25 mM imidazole, pH 7.5, 1 mM EDTA, and 2 mM RbCl or in one lacking the Rb⁺ for experiments to be conducted without Rb⁺.

**Gel Electrophoresis and Transfer to PVDF Paper—**Tricine/SDS-polyacrylamide gel electrophoresis was done essentially according to Schagger and von Jagow (1987) using either 1.5-mm 10% gels (10% separating gel (1.5 cm) plus 1-mm 15% gels (1.5 cm) or 1-mm 15% gels (1.5 cm) or 1-mm 15% gels (1.5 cm). Aluminum plates were attached to the glass plates of 16.5% gels to equalize local heat production. The following modifications were implemented to maximize radiolabeled modification of polypeptides (Moos et al., 1988). 1) Samples were dissolved in stock buffer (diluted 5-fold) containing 0.313 mM Tris, 10% SDS, 50% sucrose, 0.025% Serva Blue G, 10% mercaptoethanol, and 50 mM glutathione. 2) Gels were gelled for about 24 h. 3) A prerun was performed for 1 h at 25 mA using 5-fold diluted sample buffer and cathode buffer containing 1 M Tris, pH 8.45, 0.1% SDS, and 10% glycerol. 4) Thioglycolate (0.1 mM) was added to the running cathode buffer. Gels were run for 2 h at 30 mA (16.5%) or 12 mA (10%) and fixed and stained as described (14).

Electroblotting from unfixed and unstained 10% gels onto PVDF paper followed the procedure of Matsudaira (1987) using a Semi-Phor semi-dry transfer apparatus (Hoefer Scientific Instruments). Transfer was essentially complete after 1 h at 12 V. The PVDF paper was stained as described by Xu and Shively (1988), washed in water, and air-dried. To transfer peptides efficiently from 16.5% gels to PVDF paper, stained bands (four to five lanes) were cut out of the gel and placed horizontally in wells two or three lanes wide of a second 10% gel; gel buffer was added; and the gel was run overnight and then transferred to PVDF paper the next morning.

Before application to gels, samples were delipidated unless stated otherwise. Membranes were dissolved in 2% SDS and then 4 volumes of ice-cold methanol were added. After −0.5 h at 0 °C (or preferably −20 °C to −30 °C for overnight storage) the precipitate was collected by centrifugation at 12,000 × g for 20 min in a Sorvall centrifuge, dried in a stream of nitrogen, and dissolved in gel buffer. About 20% of the protein was lost in the precipitation procedure.

**Separation of Peptides by Size-exclusion HPLC—**The HPLC system consisted of a Rheodyne 7125 injector, a Waters 401 pump, a Waters 441 absorbance detector operating at 280 nm, and an Omniscribe series 5000 recorder. Two columns of TSK-G3000SW (7.5 mm, internal diameter, ×30 cm) connected in series, with a TSK-SW guard column (7.5 mm, internal diameter, ×7.5 cm), were used for separation (see Järsgen and Collins (1986)). Delipidized trypsin-digested enzyme was centrifuged, and the pellet was dried as described above and redisolved by vortexing for 15 min in a small volume of 2% SDS at a protein concentration of 3–4 mg/ml. Samples were centrifuged on a Beckman Airfuge for 20 min at 30 psi to remove any undissolved material, and clear solution containing up to 1 mg of protein was injected onto the TSK column. Protein was eluted at a flow rate of 0.2 ml/min with a solution of 100 mM sodium acetate, pH 4.5, containing 0.5% SDS (the pressure always being <300 psi). Fractions were collected manually. Four volumes of ice-cold methanol were added, and protein was collected by centrifugation as described above and subsequently dried.

**Sequencing—**An Applied Biosystems Model 475A Protein Sequencer with an on-line Model 120A phenylthiohydantoin analyzer was used. Strips of PVDF paper with fragments from four or five lanes of gels run with 50–100 µg of protein/lane were processed together, yielding 10–70 pmol of amino acid per cycle.

**ImmunobLOTS—**Immunoglobulins raised against the synthetic peptide ETTY and affinity-purified as described by Bayer (1990) were supplied by Dr. Jack Kyte (Chemistry Department, University of California at San Diego, La Jolla, CA) as a precipitate in 50% saturated ammonium sulfate. Suspension (2 ml) was dialyzed against 10 volumes of saline (0.4 M NaCl, 10 mM Tris, 0.5 M NaCl). From absorbance at 280 nm, the protein concentration was estimated to be −0.13 mg/ml. The antibody was diluted 1:100 or 1:400 in a solution of 1.5% (w/v) bovine serum albumin in TBS and used for immunoblotting.

**Immunoblots—**Immunoglobulins raised against the synthetic peptide ETTY and affinity-purified as described by Bayer (1990) were supplied by Dr. Jack Kyte (Chemistry Department, University of California at San Diego, La Jolla, CA) as a precipitate in 50% saturated ammonium sulfate. Suspension (2 ml) was dialyzed against 10 volumes of saline (0.4 M NaCl, 10 mM Tris, 0.5 M NaCl). From absorbance at 280 nm, the protein concentration was estimated to be −0.13 mg/ml. The antibody was diluted 1:100 or 1:400 in a solution of 1.5% (w/v) bovine serum albumin in TBS and used for immunoblotting.

**Intracellular and/or Pronase-digested enzyme** were separated on 10% Tricine gels and electroblotted onto nitrocellulose paper as described by Gershoni (1988) using transfer
buffer consisting of ice-cold 16 mM Tris, 120 mM glycine, and 20% methanol. The transfer was performed at 4°C for 3 h at 80 V. The nitrocellulose paper was washed for 10 min in TBS, quenched for 1 h in a solution of 3% (w/v) bovine serum albumin dissolved in TBS, incubated with anti-ETYY for 2 h at 22°C, and then washed four times for 5 min in TBS solution with added 0.05% Tween 20 (TTBS).

The paper was then incubated for 1 h with goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad) diluted 1:3000-fold in 1.5% (w/v) bovine serum albumin in TBS and washed again three times in TTBS and once in TBS solution, and finally the horseradish peroxidase color development reagent was added. The reagent consists of 120 mg of 4-chloronaphthol in 40 ml of ice-cold methanol mixed with a fresh solution of 72 µl of 50% hydrogen peroxide in 200 ml of TBS. After ~15 min, the reagents were washed away with distilled water.

Materials—For SDS-polyacrylamide gel electrophoresis and immunoblotting, all reagents were of electrophoresis grade from Bio-Rad. TPCK-trypsin (bovine pancreas), choline chloride (recrystallized from hot ethanol), and Dowex 50-X8 (100 mesh) were from Merck. Pronase E and proteinase K were obtained from Boehringer Mannheim. Soybean trypsin inhibitor, thiglycolate, and low molecular mass standards (2.5-16.9 kDa) were from Sigma. PVDF paper was from Millipore. *RbCl was from Amersham Corp.

RESULTS

Digestion with Trypsin, Pronase, or Proteinase K—Previously, we described the conditions for extensive tryptic digestion at pH 8.5 with retention of Rb⁺ occlusion (Karlish et al., 1990). The α chain was digested to a 19-kDa fragment and smaller less well-resolved fragments, and the β chain was essentially intact. Digestion at lower pH values (pH 7.0 and 6.5) showed that the β chain can be split almost quantitatively into two fragments of ~50 and 16 kDa, and occlusion is still retained. Peptides of 10 kDa or less were not well resolved in the 10% gel. The 16.5% gel of FIG. 1 shows fragments remaining in the membrane after treatment of Na⁺,K⁺-ATPase with TPCK-trypsin (lane T), TPCK-trypsin and then Pronase (lane TP), Pronase (lane P), proteinase K (lane PK), and proteinase K and then Pronase (lane PKP).

Resection of peptides at the lower molecular mass range required the use of the 16.5% gels and delipidation of the samples (see "Experimental Procedures"). Preliminary experiments were performed to determine the maximal time of digestion permitting retention of Rb⁺ occlusion, with each lane in FIG. 1 representing the products of such digestions. Typical specific activities of Rb⁺ occlusion for these digested membranes are given in Table I.

Fig. 1 (lane I) shows the membrane-bound fragments after extensive tryptic digestion at pH 7.0. The α chain has totally disappeared, although this is not easily seen here since the intact α chain does not enter the 16.5% gel. What is left? Note the remaining β chain and its two fragments of ~50 and 16 kDa, the prominent 19-kDa fragment of the α chain, and, in addition, six or seven peptides of 7.6-11.7 kDa. 48% of the protein was solubilized. Thus, the specific activity of occlusion rose ~2-fold (Table I). The trypsinized enzyme 19-kDa membranes was subjected to Pronase (1.3 enzyme:Pronase, w/w) for 1 h (FIG. 1, lane TP). The β chain and the 50-kDa fragment disappeared; the 19-kDa fragment was slightly shortened to 18.5 kDa; the 16-kDa β fragment disappeared; and a fragment of 13 kDa appeared. There are three relatively prominent smaller peptides of 7-9 kDa and also many poorly resolved minor bands, which is not surprising in view of the lack of specificity of the Pronase. About 90% of the initial membrane protein was removed, and the specific activity of occlusion therefore rose by 2.5-fold. In other experiments, as much as 70% of the protein was solubilized, and the specific activity rose over 3-fold. Essentially the same specific activity was achieved by treating the 19-kDa membranes with proteinase K (Table I). Fig. 1 (lanes P, PK, and PKP) shows the peptides remaining in the membrane after treatment of Na⁺,K⁺-ATPase only with the nonselective proteases. Pronase alone (lane P) (1:10 enzyme:Pronase, w/w) removed ~50% of the membrane protein including the bulk of both the α and β chains without destroying Rb⁺ occlusion (Table I). Left in the membrane were two clearly distinguishable bands at 21 and 19 kDa, a band at 15 kDa, and many other minor bands. In some experiments using a higher ratio of enzyme to Pronase or those in which the digested pellet was stored overnight at 4°C, the 21-kDa band was not detected (see FIG. 2). Thus, the 21-kDa fragment is more susceptible to Pronase digestion than is the 19-kDa fragment, and an intact 21-kDa fragment is not essential for occlusion. Treatment of the Na⁺,K⁺-ATPase with proteinase K (lane PK) (1:20 enzyme:proteinase, w/w) released ~55% of the protein without destroying occlusion (Table I) and left in the membrane a fragment of 28 kDa, a doublet of bands at 19 and 18.5 kDa, a 15-kDa band, and poorly resolved smaller fragments. Finally, in the double digestion of proteinase K and then Pronase (lane PKP), the 28-kDa fragment disappeared; and of the doublet, only the 18.5-kDa fragment remained, and >60% of the original protein had been removed from the membrane.

| Conditions | Rb⁺ occlusion (nmol/mg protein) |
|------------|-------------------------------|
| Control    | 3.5                           |
| Trypsin    | 6.70                          |
| Trypsin then Pronase | 8.74                        |
| Trypsin then proteinase K | 8.84                      |
| Pronase    | 7.28                          |
| Proteinase K | 7.60                       |
| Proteinase K then Pronase | 8.99                      |
Proteolysis of Na\textsuperscript{+},K\textsuperscript{+}-ATPase with Preservation of Rb\textsuperscript{+} Occlusion

Fig. 2. Cation specificity of trypsin and Pronase digestion. Na\textsuperscript{+},K\textsuperscript{+}-ATPase (250 µg) was digested for 1 h at 37 °C with trypsin (1:7, w/w) or Pronase (1:20, w/w) in medium containing 10 mM Tris, pH 8.5, 1 mM EDTA, and with an additional 10 mM Tris, RbCl, or NaCl as indicated. The digestion was stopped as indicated under “Experimental Procedures,” and samples were taken for measurement of protein and Rb\textsuperscript{+} occlusion (Table II) and the 16.5% gel. In this experiment, samples were not delipidated, but digested membranes from equivalent amounts of control enzyme (≈100 µg) were applied to each lane.

Table II

| Condition | Cation | Rb\textsuperscript{+} occlusion |
|-----------|--------|-------------------------------|
| Control   |        | 3.72 nmoI/mg protein          |
| Trypsin   | Tris   | 0.39                          |
| Trypsin   | Rb\textsuperscript{+} | 7.15                          |
| Trypsin   | Na\textsuperscript{+} | 5.83                          |
| Pronase   | Tris   | 1.97                          |
| Pronase   | Rb\textsuperscript{+} | 7.84                          |
| Pronase   | Na\textsuperscript{+} | 7.74                          |

In experiments using proteinase K, it was necessary to treat the digested membranes with phenylmethylsulfonyl fluoride, to wash three times, and then to precipitate the protein by the SDS/methanol method to observe the relatively clear fragmentation patterns. If these precautions were not taken, only nonreproducible patterns were observed due probably to the persistence of traces of active proteinase K in the gel samples and a slow but continuing digestion of the solubilized peptides.

An obvious conclusion from these experiments is that the bulk of neither the α nor β chains is required for Rb\textsuperscript{+} occlusion, full activity being maintained even in membranes containing no peptide larger than 18.5 kDa (Fig. 1, lane TP or PKP). The membrane-embedded parts of the protein remain intact.

The remarkable resistance of the 19-kDa (or 18.5-kDa) fragment to further digestion by either trypsin or the nonselective proteases depends critically on the conditions. As reported previously for proteinase, the absence of Ca\textsuperscript{2+} was obligatory, as was the presence of Rb\textsuperscript{+} ions (Karlish et al., 1990). In the case of Pronase or proteinase K, the divalent cation Mg\textsuperscript{2+} or Ca\textsuperscript{2+} must also be absent (data not shown).

The monovalent cation requirement for both tryptic and Pronase digestion is critically dependent on the conditions. As reported previously for trypsin, the absence of Ca\textsuperscript{2+} was obligatory, as was the presence of Rb\textsuperscript{+} ions (Karlish et al., 1990). In the case of Pronase or proteinase K, the divalent cation Mg\textsuperscript{2+} or Ca\textsuperscript{2+} must also be absent (data not shown).

Table III documents the N-terminal sequences of peptides with apparent molecular masses of 13 kDa or larger. Of the α chain fragments, the major 19-kDa tryptic fragment begins at residue Asn\textsuperscript{830} and extends toward the C terminus, as reported.
previously (Karlish et al., 1990). Treatment of the trypsin-digested membranes with Pronase (1:3 enzyme:Pronase, w/w) cuts off 3 residues from the N terminus of the 19-kDa fragment, with the resultant 18.5-kDa fragment beginning at Thr\(^{195}\). Digestion of Na\(^+\),K\(^+\)-ATPase with Pronase alone (1:10 enzyme:Pronase, w/w) produces a 19-kDa fragment beginning also at Asp\(^{831}\) and the 21-kDa fragment with N-terminal Lys\(^{380}\), which is digested further relatively easily.

Of the \(\beta\) chain trypsin fragments, the major 16-kDa and minor 14-kDa fragments are located near the N terminus at Ala\(^{3}\) and Phe\(^{14}\), respectively. Treatment of trypsin-digested enzyme with Pronase cuts off a further 15 residues from the 16-kDa fragment, producing the 13-kDa fragment with N-terminal Lys\(^{380}\). These fragments do not contain glycosylation sites and therefore run as sharp bands on the gel. The diffuse 50-kDa peptide starting at Gly\(^{143}\) of the \(\beta\) chain contains all putative glycosylation sites. Proteolytic digestion of the \(\beta\) chain into two fragments with papain (Chin, 1985) or chymotrypsin (Farley et al., 1986) has been reported before. The larger and smaller fragments were assigned to the N- and C-terminal halves of the chain, respectively (see also Brown et al. (1987)), which appear opposite to the present assignments; but N-terminal sequences of the two fragments were not determined.

Fig. 3A shows separations of SDS-solubilized 19-kDa membranes into families of peptides by size-exclusion HPLC on a TSK-3000 column under nonreducing conditions. Four peaks of absorption at 280 nm were cleanly resolved. Protein in each fraction was precipitated by addition of 4 volumes of ice-cold methanol, and samples were applied to a 16.5% Tricine gel (Fig. 3B), i.e., lanes 1–4, and, for comparison, an equivalent sample of the original 19-kDa membrane (lane 7). The comparison shows that recovery of protein from the TSK column is essentially quantitative. Fraction 1 contains a mixture of high molecular mass protein impurities. Fraction 2 contains the intact \(\beta\) chain as well as both the 50- and 16-kDa fragments. The \(\approx 50\)- and 16-kDa fragments are connected by an S–S bridge and therefore behave exactly like the intact \(\beta\) chain on the column, but separate under the reducing conditions of the gel (see "Discussion"). Fraction 3 consists mainly of the 19-kDa fragment, close to one-half pure as judged by scans of Coomassie Blue in the lane. Fraction 4 is a mixture of the seven peptides of 7.6–11.7 kDa. The far right-hand lane shows concentrated peptides from fraction 4 of another experiment, next to marker peptides (lane M). These peptides, a–g, were used for sequencing (Table IV).

Peptides a–c (11.7, 11.3, and 10.9 kDa, respectively) all have the same N terminus (Asp\(^{86}\)) and therefore differ in length at the C terminus. These fragments are thought to contain transmembrane segments M1 and M2. Prominent peptide d (9.5 kDa) gave no sequence in two separate experiments. Conceivably, it is the so-called \(\gamma\) chain (see Forbush (1983) and Collins and Leszyk (1987)) because a peptide in the control enzyme runs near this position\(^2\) and may well be both

\(^2\) J. M. Capasso, S. Hoving, D. M. Tal, R. Goldshleger, and S. J. D. Karlish, unpublished data.
resistant to trypsin and blocked at the N terminus, thus precluding sequencing. Peptide e (8.7 kDa), beginning at Ile^263^, is thought to contain transmembrane segments M3 and M4. Peptide f (8.0 kDa), beginning at Gln^257^, is thought to contain transmembrane segments M5 and M6. Peptide g (7.6 kDa) gave a sequence that could not be recognized in either the α or β chain. In earlier experiments in which the Na^+^-K^+^-ATPase was digested with trypsin rather than TPCK-trypsin, fragments beginning at Ala^264^ (segments M3 and M4) and Ser^270^ (segments M5 and M6) were identified. These appear to be the result of chymotryptic splits. Digestion with TPCK-trypsin rather than trypsin led to clean sequencing results without complications due to secondary sequences from overdigestion. Rather than trypsin, TPCK-trypsin was digested with trypsin rather than TPCK-trypsin, and the resulting digestion with TPCK-trypsin gave a sequence that could not be recognized in either the N-terminal Thr^2^ or the C-terminal Thr^9^.

Attempts to sequence the smaller fragments in the Pronase-digested membranes were successful only in the case of the prominent peptide of 7.8 kDa (Fig. 1, lane TP, α). This turns out to be a fragment of the 18.5-kDa peptide with the same N-terminal Thr^2^. This result was surprising since it suggests that an intact 19- or 18.5-kDa peptide may not be absolutely essential for Rb^+^ occlusion to be maintained. Further support was obtained in the experiments of Fig. 4. All Pronase fragments yet observed are "tryptic" splits in that the N termini of the higher molecular mass of the 19-kDa fragment is therefore 21,821 Da, whereas that of the 18.5-kDa fragment is 21,482 Da.

**DISCUSSION**

Digestion with Nonselective Proteases Compared to That with Trypsin—Digestion with the nonspecific proteases Pronase and Proteinase K removes up to 70% of the membrane protein without destroying Rb^+^ occlusion. This clearly strengthens the previous conclusion that the Rb^+^ occlusion sites lie within transmembrane segments (Karlish, 1990). High affinity Ca^2+^-binding sites of sarcoplasmic reticulum Ca^2+^-ATPase are also suggested to lie within transmembrane segments, based on site-directed mutagenesis experiments (Clarke et al., 1989, 1990). Digestion with Pronase and Proteinase K also demonstrates that the bulk of the β chain is unnecessary for maintenance of Rb^+^ occlusion. Finally, the results with Pronase and proteinase K prove that the resistance of the 19-kDa fragment to further digestion is not due to peculiar requirements of specificity of the proteolytic enzyme or even to a protective interaction with the intact β chain. Rather, one must conclude that all the proteolytic enzymes are sterically hindered from approaching and cutting 

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**Fig. 4. Binding of anti-ETYY antibody to intact α chain and 19-kDa fragment.** Upper, delipidated control enzyme (35 μg) or tryptic 19-kDa membranes (40 μg) were applied to lanes of a 10% gel, and peptides were transferred to nitrocellulose that was immunoblotted with 1:100-, 1:400-, and 1:1600-fold dilutions of the anti-ETYY antibody. Shown are the lanes blotted with the 1:400-fold dilution. Lower, 70 μg of delipidated tryptic 19-kDa membranes or such membranes incubated with Pronase under the conditions of Fig. 1, i.e. trypsin then Pronase, was used for the gel and immunoblot with anti-ETYY at a 1:400-fold dilution. Lane M, low molecular mass standards.
the polypeptide chain by its association with the lipid. Nevertheless, Pronase removes 3 residues from the N terminus of the 19-kDa fragment and can at least partially split the chain (Table IV) without loss of Rb+ occlusion (Fig. 1 and Table I); thus, segments within the aqueous phase are at least somewhat accessible to Pronase.

Resistance of the 19-kDa fragment to either trypsin or Pronase requires the presence of cations that bind to the monovalent cation center, i.e. K+ or Na+ and their congeners (Fig. 2 and Table II). Binding of either Na+ or K+ could induce a more compact structure in which connecting polypeptide loops or free ends within the aqueous phase become tightly associated with the membrane surface or embedded within the lipid. This finding supports the notion that K+ and Na+ bind to the same sites (see also Shani-Sekler et al. (1988)).

The protection of the 19-kDa fragment by either K+ or Na+ and congeners can be contrasted with the well-known phenomenon that binding of K+ or Na+ to native enzyme induces different and distinct tryptic (Jørgensen, 1975) and chymotryptic (Jørgensen and Petersen, 1985) digestion patterns. These initial splits of the α chain reflect a differential sensitivity to trypsin or chymotrypsin of particular bonds in the cytoplasmic domain. Thus, the peptide products of extensive digestion in the presence of K+ or Na+ are the same, but the route taken is different.

**Proteolytic Fragments of the α Chain Involved in Rb+ Occlusion**—Having identified proteolytic fragments containing all of the transmembrane segments of the α chain (Tables III and IV), one may ask, "What are the minimal lengths of peptides compatible with or/and involved in Rb+ occlusion?" One criterion is that any fragment that can be cut without loss of occlusion is not required intact, although remaining fragments may be involved. Conversely, any fragment that is cut with loss of occlusion must be essential, although it does not necessarily bind cations directly. Previously, we demonstrated a strict correlation between the amount of the 19-kDa fragment and Rb+ occlusion when the trypsinized membranes are digested further in the presence of Rb+ and Ca2+ ions (Karlish et al., 1990). Thus, at least part of the 19-kDa fragment is essential. On the other hand, the finding of smaller fragments of the 18.5-kDa peptide in trypsinized membranes digested further with Pronase but still able to occlude Rb+ (e.g. Fig. 1 and Table IV) shows that the entire 19-kDa polypeptide cannot be crucial. Of the other smaller tryptic peptides (Fig. 3 and Table IV), the minimal sizes compatible with preservation of Rb+ occlusion are obviously peptide e (10.7 kDa, beginning at Asp56), peptide e (8.7 kDa, beginning at Ile89), and peptide f (8.0 kDa, beginning at Gln173). In experiments using trypsin rather than TPCK-trypsin, we found an even shorter peptide of 7.0 kDa corresponding to peptide e and beginning at Ser56 (Karlish et al., 1991). Pronase can apparently digest the small tryptic peptides still further (Fig. 1, compare lanes T and TP), but the resultant peptides are not resolved well enough to permit sequencing. The peptide pattern of TPCK-trypsin-treated membranes is the cleanest and most reproducible, but the minimal peptide lengths compatible with occlusion are actually somewhat shorter.

Peptide g in Fig. 3B (sequence DLGGSAPLAIGFS) was not identified in either the α or β chain. Presumably, it is a contaminant. Partially homologous sequences were found in human apolipoprotein C-III (XLGGGWPLGXXGA) or hobo apolipoprotein E (DLGLQPLPLGKFR).

What is the evidence that the 19-kDa segment or segments of it or the other tryptic fragments of the α chain are directly involved in occlusion? Recent studies of inactivation and labeling of 19-kDa membranes by dicuclohexylcarbodiimide have shown that two molecules of DCCD are incorporated at full inactivation of Rb+ occlusion, i.e. each Rb+ site contains one carboxyl group (Goldshleger et al., 1990; Karlish et al., 1991). On the basis of these and previous studies of DCCD inactivation kinetics (Shani-Sekler et al., 1988), we propose that two Na+ and K+ sites are the same, but the third Na+ site contains only neutral ligating groups. The 19-kDa fragment is specifically labeled in an Rb+-protected manner with an estimated stoichiometry of one DCCD molecule/19-kDa chain. It is now known that Glu593 is specifically labeled with DCCD (Karlish et al., 1992). A second tryptic peptide of ~9 kDa is also labeled specifically. A question arose whether the labeled 9-kDa fragment may itself be a fragment of the 19-kDa peptide. An unequivocal answer of "no" can now be given since no fragments of 19 kDa are found in trypsinized membranes (see Figs. 3 and 4 and Table IV) (unlike the situation with Pronase-treated membranes). The identity of this peptide is as yet unknown. The suggestion is that transmembrane segments of the C terminal 19-kDa fragment and at least one of the smaller limit tryptic peptides (representing M1 + M2, M3 + M4, or M5 + M6), contribute carboxyl residues and/or other ligating groups to a cation occlusion "cage."

**Arrangement of Tryptic Fragments in 19-kDa Membranes**—To build a model of the cation occlusion site, it is necessary to know the arrangement of the transmembrane segments in the plane of the membrane. However, a conceptual problem arises since the chain folding is uncertain in the critical C-terminal sector (see Jørgensen and Andersen (1988)). Hydrophobicity plots are ambiguous in this domain, and independent techniques to determine chain folding have produced conflicting results. Several models differing in the number and orientation of transmembrane segments in the C-terminal half have been proposed. Location of four transmembrane segments in the N-terminal half is not controversial. The models of Shull et al. (1986) and Kawakami et al. (1985) for the α chain of Na+,K+-ATPase predict eight and six transmembrane segments, respectively, with four and two in the C-terminal domain, respectively, and a cytoplasmically oriented C terminus, whereas that of Ovchinnikov et al. (1986) proposes seven transmembrane segments, with three in the C-terminal domain and an extracellular C terminus. The model of the homologous sarcoplasmic reticulum Ca++ pump proposes 10 transmembrane segments, with six in the C-terminal domain and a cytoplasmic C terminus (MacLennan et al., 1986). Since hydrophathy profiles of the different pumps are similar, it is not likely that their topology is different.

Fig. 5 shows four possibilities for the folding of the trypptic peptides of the α chain in 19-kDa membranes based on the seven-, two variants of the eight-, and the 10-transmembrane segment models. For each peptide, the N terminus is that found experimentally. With the exception of the 19-kDa fragment of known length, the designated C terminus of the other peptides is a guess based on the sequence and the apparent molecular mass. The actual lengths of these fragments may be a little different.

We point out here some salient features of the models. 1) Note first the different predictions concerning the orientation of the N and C termini of the 19-kDa fragment. In models B–D in Fig. 5, the C terminus is cytoplasmic, whereas in model A, it is extracellular. Studies of the binding of C-terminal specific antibodies to whole cells (extracellular) (Ovchinnikov et al., 1988) or kidney vesicles (cytoplasmic) (Kramer et al., 1990) have given contradictory results for Na+,K+-ATPase. However, very recent labeling studies show conclusively that...
FIG. 5. Possible arrangements of tryptic fragments in 19-kDa membranes. Experimentally determined N-terminal and presumed C-terminal residues, as well as the likely residue at the point of entry and exit of each transmembrane segment are indicated. The thick black dots locate the position of Glu297 in M4 and Glu313,314 in the final or penultimate transmembrane segment.

the C terminus is cytoplasmic (Modyanov, 1992). The C terminus of the related sarcoplasmic reticulum Ca2+-ATPase (Mathews et al., 1989), cell membrane Ca2+-ATPase (Strehler, 1991), and gastric H+/,K+-ATPase (Sachs et al., 1992) is cytoplasmic. In models A–C, the N terminus is extracellular, whereas in model D, it is cytoplasmic. An experiment is now being conducted to define the orientation of the N-terminal Asn894 of the 19-kDa fragment by tryptic digestion in intact right-side-out kidney vesicles. A tryptic fragment from the H+/,K+-ATPase beginning with the sequence LVNE is produced at the cytoplasmic surface (Sachs et al., 1991). The location of the sequence LVNE in H+/,K+-ATPase is close to the location of the N terminus of our 19-kDa fragment (NPKTDKLVNE); thus, the finding is suggestive of a 10-transmembrane segment model for Na+,K+-ATPase.

2) The resistance of the 19-kDa fragment to further proteolytic digestion is easiest to understand on the basis of models with the maximal number of transmembrane segments (e.g. Model D) since, in general, one may expect long loops or tails of the protein in the aqueous phase (e.g. as in Model A) to be cut. A rough measure of the lengths of protein in the aqueous phase that are inaccessible to proteolytic enzymes can be obtained by inspection of the location of N termini of tryptic fragments in the full primary sequence. A striking point (see Fig. 5 and Tables III and IV) is that the N-terminal residue of tryptic fragments is usually not at the theoretical tryptic limit immediately preceding predicted transmembrane segments. Apparently, trypsin or other proteases cannot approach closer than 10–20 residues from the membrane and shave away more protein. The finding that neither trypsin nor Pronase was able to remove the C-terminal residues ETYY (Fig. 4) was particularly surprising since the preceding sequence RKLIRRR beginning at residue 998 could be expected to be readily cut. This finding is easiest to reconcile with model C or D, in which the peptide tail emerging from the final transmembrane segment is only 22 residues long and is hindered from reaction with trypsin or Pronase.

3) There is some prior information in the literature that appears to be inconsistent with certain models. Ovchinnikov et al. (1987) have reported the presence of two water-soluble tryptic fragments corresponding to hydrophobic sequences 909–930 and 973–994. This surprising finding is seemingly incompatible with models B–D. In addition, these authors have concluded that monoclonal antibodies to peptide regions 810–825 and 887–904 bind to extracellular and cytoplasmic epitopes, respectively (Ovchinnikov et al., 1988). This also appears incompatible with model D.

Evidently, more critical experimental data for Na+,K+-ATPase are required to definitively exclude particular models and to build reliable models of a cation occlusion and binding path. It one accepts the view that the topology of the related pumps is likely to be similar, the 10-transmembrane segment model (MacLennan et al., 1985) appears to be the most favored option.

Role for β Subunit in Cation Occlusion?—An unexpected result is that the 16-kDa fragment of the β chain is protected against further digestion by the presence of Rb+ or Na+ ions (Fig. 2). The same phenomenon is seen less clearly for the Pronase digestion (Fig. 2). The 16-kDa fragment, extending from Ala65 probably to Arg142 (molecular mass of 16,039 Da), contains the single putative transmembrane segment and none of the glycosylation sites. Can this finding be taken to mean that this segment of the β chain is essential for maintenance of occlusion and may actually be a component of the occlusion cage? Neither of these possibilities can be excluded. However, by analogy with pumps that lack a β subunit, e.g. Ca2+ and H+ pumps, a direct role of the β chain may seem unlikely. Also based on radiation inactivation studies, we have suggested that the cation-binding sites and transport path of the Na+/K+ pump are located exclusively on the α chain (Karlish and Kempner, 1984). Perhaps the transmembrane segment of the 16-kDa unit interacts with those of the α chain.

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2 J. Kyte, personal communication.
3 S. J. D. Karlish and P. L. Jørgensen, unpublished data.
that constitute the occlusion path, sense the binding of the cations indirectly, and transmit the "conformational" information to the relevant trypsin-sensitive bond(s). In this case, symmetry of effects would require that interactions of the transmembrane segment of the β chain with those of the α chain affect cation occlusion, e.g. an apparent cation affinity.

The second β fragment (≈50 kDa, beginning at Gly143 and probably extending to the C-terminal Ser552) is linked to the 16-kDa fragment via an S-S bridge between Cys138 and Cys146 and contains all the known glycosylation sites and two additional S-S bridges (Kirley, 1989; Miller and Farley, 1989, 1990). The protein component has a probable molecular mass of 18,506 Da, and sugars compose ≈30 kDa. The β chain is thought to be divided into two rather compact domains (McDonough et al., 1990). Trypsin cuts the protein near the predicted hinge to produce the ≈50- and 16-kDa fragments, and the same is probably the case for papain (Chin, 1985) or chymotrypsin (Farley et al., 1986). The Pronase digestions (Fig. 1) show that the ≈50-kDa fragment can be removed without affecting occlusion; however, there is a final point of interest. The ≈50-kDa fragment has not been thought to contain transmembrane domains. But attempts to reduce the Cys138-Cys146 S-S bridge with high concentrations of mercaptoethanol and then to wash away the ≈50-kDa fragment were not successful, even when using conditions considered to be suitable for removing "extrinsic" membrane proteins such as high salt concentration or sodium carbonate. On the other hand, this fragment is heavily labeled with the hydrophobic phase label 3-trifluoromethyl-3-(m-131Iodophenyl)diizirine. It may therefore be possible that, in reality, the C-terminal half of the β chain is anchored in the membrane by a transmembrane segment.

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