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A 19-kDa C-terminal tryptic fragment of the α chain of Na/K-ATPase is essential for occlusion and transport of cations

(selective proteolysis/cation sites/energy transduction mechanism)

S. J. D. KARLISH*†, R. GOLDSHLEGER*, AND W. D. STEIN‡

*Biochemistry Department, Weizmann Institute of Science, Rehovot, Israel; and ‡Institute of Life Science, Hebrew University, Jerusalem, Israel

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ABSTRACT **Tryptic digestion of pig renal Na/K-ATPase in the presence of Rb and absence of Ca ions removes about half of the protein but leaves a stable 19-kDa membrane-embedded fragment derived from the α chain, a largely intact β chain, and essentially normal Rb- and Na-occlusion capacity. Subsequent digestion with trypsin in the presence of Ca or absence of Rb ions leads to rapid loss of the 19-kDa fragment and a parallel loss of Rb occlusion, demonstrating that the fragment is essential for occlusion. The N-terminal sequence of the 19-kDa fragment is Asn-Pro-Lys-Thr-Asp-Lys-Leu-Val-Asn-Glu-Arg-Leu-Ile-Ser-Met-Ala, beginning at residue 830 and extending toward the C terminus. Membranes containing the 19-kDa fragment have the following functional properties. (*i*) ATP-dependent functions are absent. (*ii*) The apparent affinity for occluding Rb is unchanged, the affinity for Na is lower than in the control enzyme, and activation is now strongly sigmoidal rather than hyperbolic. (*iii*) Membranes containing the 19-kDa fragment can be reconstituted into phospholipid vesicles and sustain slow Rb–Rb exchange. Thus the transport pathway is retained. We conclude that cation occlusion sites and the transport pathway within transmembrane segments are quite separate from the ATP binding site, located on the cytoplasmic domain of the α chain. Interactions between cation and ATP sites, the heart of active transport, must be indirect—mediated, presumably, by conformational changes of the protein.**

The Na/K-ATPase or Na/K-pump has been purified, cloned, and sequenced (1, 2). Much is known about transport modes, ion occlusion, and conformational changes (3). Nevertheless, the central question—how the free energy of hydrolysis of ATP is transduced into active pumping of cations—remains unanswered. ATP-binding residues are located on the central cytoplasmic loop of the α chain (1, 2), but little is known about the cation binding sites or pathway for cation movement (for a review, see ref. 4). Information on the cation binding sites would undoubtedly clarify the mechanism of coupling. In particular, by knowing whether the cation and ATP sites were adjacent or quite separate, one could distinguish between more or less direct mechanisms.

One approach to identifying cation-binding residues is chemical labeling. Carboxyl residues are good candidates, and hence reagents such as *N,N'*-dicyclohexylcarbodiimide (DCCD) have been used extensively (5–8). We (8, 9) have studied inactivation by DCCD of Rb and Na occlusion and concluded that carboxyl residues are involved and Rb and Na bind to the same residues. Incorporation of about 2 mol of DCCD per mol of α chain accompanies full inactivation of Rb occlusion (10). Subsequent attempts to locate the label in the primary sequence could exploit selective proteolysis (11). In control experiments designed to produce extensive digestion, we were surprised to find a condition in which occlusion was retained while the α chain was largely but incompletely fragmented. In this condition a stable 19-kDa membrane-embedded fragment accumulates.

This paper describes preparation of membranes containing the 19-kDa fragment, presents the N-terminal sequence of the 19-kDa fragment (which locates it in the α chain), and characterizes functional properties of these membranes. Cation occlusion and transport are retained but ATP-dependent functions are lost.

MATERIALS AND METHODS

Materials. For SDS/PAGE, molecular mass standards (10–100 kDa) and all reagents were of electrophoresis grade and from Bio-Rad. Trypsin (bovine pancreas; 24579), choline chloride (recrystallized from hot ethanol), and Dowex-50 X-8 (100 mesh) were from Merck. Soybean trypsin inhibitor (T-9003), thioglycollate (T-0632), ATP (A-5394), soybean phospholipid (P-5638), and low molecular weight standards (2.5- to 16.9-kDa SDS-17) were from Sigma. Poly(vinylidene difluoride) (PVDF) paper (IPVH 000 LO) was from Millipore. ^{86}Rb and ^{22}Na were from NEN.

Enzyme Preparations. Na/K-ATPase (specific activity, 15–21 units/mg of protein) was prepared from pig kidney and stored at −70°C, and ATPase and protein (Lowry) were assayed as described in ref. 13. Prior to use, the enzyme was dialyzed overnight at 0°C against 1000 vol of 25 mM histidine/1 mM EDTA, pH 7.0. For routine preparation of membranes containing the 19-kDa fragment (see text), Na/K-ATPase (1.5 mg/ml) was incubated for 1 hr at 37°C with trypsin (0.2 mg/ml)/10 mM Tris, pH 8.5/10 mM RbCl/0.7 mM EDTA. Tryptic inhibitor (0.85 mg/ml) was then added. The suspension was diluted with 25 mM imidazole, pH 7.5/1 mM EDTA/1 mM RbCl and centrifuged at 200,000 × *g* for 1 hr, the pellet was homogenized, diluted again, recentrifuged, resuspended in this medium, lacking RbCl, and stored on ice.

Gel Electrophoresis. SDS/PAGE using Tricine buffer was essentially as described by Schagger and Von Jagow (14). The following modifications were implemented (12): (*i*) Samples were dissolved in a stock buffer (diluted 1:5) containing 0.313 M Tris, 10% (wt/vol) SDS, 50% (wt/vol) sucrose, 0.025% Serva blue G, 10% (vol/vol) 2-mercaptoethanol, and 50 mM glutathione. (*ii*) Gels were aged for 24 hr. (*iii*) Gels were preelectrophoresed for 1 hr at 25 mA (30 min at 30 V for Mini-Gels), by using 1:5 diluted sample buffer and cathode buffer containing 1 M Tris (pH 8.45), 0.1% SDS, and 0.1 mM thioglycollate. (*iv*) Thioglycollate (0.1 mM) was added to the running cathode buffer. Full-size gels were electrophoresed for 24 hr at 16 mA (2 hr at 95 V for Mini-Gels) and fixed and stained (14). Electroblotting of polypeptides from unfixed and unstained full-size gels onto a PVDF sheet, followed the

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; PVDF, poly(vinylidene difluoride).

†To whom reprint requests should be addressed.

procedure of Matsudaira (15) using a Semi-Phor TE70 semi-dry transfer apparatus (Hoefer Instruments). Transfer was essentially complete after 3 hr at 12 V. The PVDF sheet was stained (15), washed in water, and air-dried, and bands containing the 19-kDa fragment were excised and stored at −20°C.

Sequencing. An Applied Biosystems model 475A protein sequencer with an on-line model 120A phenylthiohydantoin analyzer was used. Five or six strips of PVDF with 19-kDa fragments (from gels with 50 μg of protein per lane) were processed together, yielding 12–71 pmol of amino acids per sequencer cycle.

Functional Assays. Phosphorylation by [^{32}P]ATP and the effect of ATP on Rb occlusion were assayed (9). ATP-displaceable binding of 2′,4′,5′,7′-tetrabromofluorescein (eosin) was estimated by fluorescence (8). ^{86}Rb occlusion and ^{22}Na occlusion stabilized by oligomycin were assayed manually at 20°C (8). The standard Rb occlusion assay medium (40 μl) contained 30 μg of control or proteolyzed enzyme, 0.025–5 mM ^{86}Rb (3–8 × 10^5 cpm) and Tris·HCl (pH 7.0) or choline chloride to a total of 100 mM. Dissociation of ^{86}Rb at 20°C was measured by mixing 20 μl of enzyme suspension, 0.25 mM ^{86}Rb, and 20 μl of medium containing RbCl or NaCl (final concentration, 20 mM or 75 mM, respectively), and, at 0–120 sec, 500 μl of ice-cold sucrose, etc., was added. The standard Na occlusion assay medium (40 μl) contained 50 μg of control or proteolyzed enzyme, oligomycin (75 μg/ml), 0.04–20 mM NaCl, ^{22}Na at 3–5 × 10^6 cpm, and choline chloride to 100 mM. Elution and measurement procedures were as described (8, 9).

Reconstitution. Membranes containing the 19-kDa fragment were reconstituted into soybean phospholipid vesicles by freeze–thaw–sonication, essentially as described for control enzyme (16), using lipid prepared as described (17), but slightly modified as follows for optimal reconstitution: (*i*) lipid/protein, 40:1 (wt/vol), (*ii*) protein/cholate, 1:1 (wt/wt), and (*iii*) 5-sec sonication using a Bransonic 12 bath sonicator. The external medium of vesicles loaded with 150 mM RbCl was then exchanged for 150 mM choline chloride/25 mM histidine, pH 7.0/1 mM EDTA (see ref. 17). For assay of Rb–Rb exchange (16), vesicles (≈100 μl) were mixed with equal volumes of the choline chloride medium containing RbCl at a final concentration of either 0.2–2 mM (low) or 20 mM (high) and a fixed amount of ^{86}Rb (1–2 × 10^6 cpm per sample). Elution of vesicles with trapped isotope on Dowex-50 columns was as in ref. 16.

Calculations. Linear and nonlinear curve fitting was performed using the program Enzfitter (Elsevier-Biosoft).

RESULTS

Fig. 1*A* shows the time course of tryptic digestion of Na/K-ATPase in a medium containing Rb ions and EDTA with roughly equimolar trypsin and Na/K-ATPase, 1:7 (wt/wt). The gel shows fragments remaining within the membrane. Within 10 sec, the α chain disappeared and, with time, successively smaller fragments appeared and disappeared. By 1 hr, a reproducible pattern emerged. The major product was a stable 19-kDa fragment, with minor 16- and 14-kDa and other, poorly resolved, smaller fragments. Only insignificant amounts of larger fragments of the α chain remained in the membrane. The β chain was largely undigested (11). Approximately 40–50% of the membrane protein was released into the medium. Other experiments (data not shown) demonstrated that the 19-kDa fragment was only slowly digested ($t_{1/2}$, ≈4 hr). Fig. 1 shows that the Rb occlusion capacity is essentially unchanged.

At the high trypsin/protein ratio used in Fig. 1*A*, one might expect complete digestion of exposed regions of the α chain. Critical conditions for producing the stable 19-kDa fragment were therefore investigated (Fig. 1*B*). Evidently, the presence of Rb and the absence of Ca are essential. In the absence of Rb, the 19-kDa fragment was largely destroyed with loss of Rb occlusion. In the presence of Ca, the 19-kDa fragment

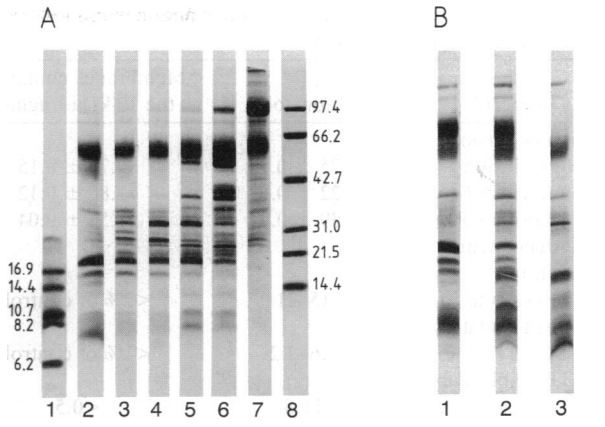

FIG. 1. Time course (*A*) and conditions (*B*) for accumulation of the 19-kDa fragment. (*A*) Na/K-ATPase (14.8 mg) was incubated with 2.1 mg of trypsin in standard Tris/RbCl/EDTA medium. Na/K-ATPase (250 μg) and 36 μg of trypsin were mixed with 107 μg of tryptic inhibitor. Rb occlusion at 5.5 mM Rb was measured in samples. The remainder was centrifuged in a Beckman Airfuge for 30 min at 30 psi (1 psi = 6.9 kPa) and washed with 25 mM imidazole/1 mM EDTA, pH 7.5, and the pellets were dissolved in 1:5 diluted gel buffer. Equal volumes of dissolved control enzyme (50 μg) and proteolyzed membranes were applied to a 10% gel. The length of incubation with trypsin and the percent of Rb occlusion are as follows. Lanes: 1 and 8, molecular mass markers (in kDa); 2, 60 min/89% of control; 3, 20 min/90% of control; 4, 5 min/82% of control; 5, 1 min/95% of control; 6, 10 sec/113% of control; 7, control. (*B*) Na/K-ATPase (1 mg) was incubated for 1 hr at 37°C with 145 μg of trypsin in medium containing 10 mM RbCl/1 mM EDTA (lane 1) or 10 mM choline chloride/1 mM EDTA (lane 2) or 10 mM RbCl/1 mM $CaCl_2$ (lane 3). Tryptic inhibitor (450 μg) was added. Rb occlusion (at 4.5 mM Rb) was measured, and the remainder was processed. Quantities of proteolyzed membranes equivalent to 50 μg of control enzyme were applied to each lane. Rb occlusion, calculated as percent of control, was as follows. Lanes: 1, 89%; 2, 16%; 3, 3%.

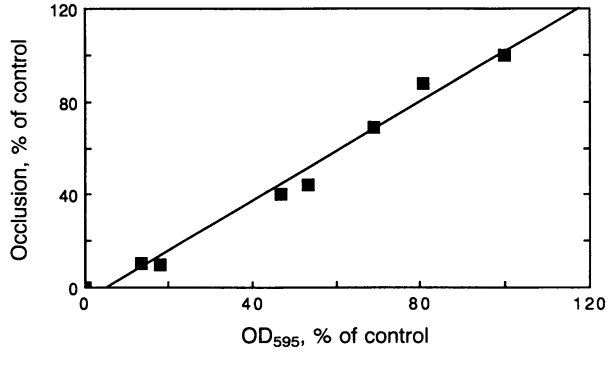

FIG. 2. Correlation between Rb occlusion and amount of the 19-kDa fragment. Membranes containing the 19-kDa fragment (1.3 mg) were incubated in medium containing 10 mM Rb and 1 mM $CaCl_2$ with 26 μg of trypsin [i.e., 1:50 (wt/wt); compare the 1:7 ratio in Fig. 1]. From 10 sec to 4 min, samples were mixed with tryptic inhibitor (45 μg), Rb occlusion was measured, and membranes were pelleted in the Airfuge and dissolved in gel buffer. Volumes equivalent to 25 μg of control enzyme were applied to a 10–20% Mini-Gel (Novex, Encinitas, CA). After electrophoresis, staining, and thorough destaining, the 19-kDa band was excised and homogenized in 1 ml of 2% SDS, and the polyacrylamide was removed by centrifugation. The absorbance of material extracted from each lane was measured at 595 nm. The absorbance of material extracted from a piece of gel outside each lane was subtracted.

Table 1. Functional properties of control and membranes containing the 19-kDa fragment

Property	Control	Membranes containing the 19-kDa fragment
Cation occlusion*		
Rb occlusion	3.75 ± 0.12	7.74 ± 0.15
Na occlusion	5.22 ± 0.22	11.86 ± 0.12
Ratio Na/Rb	1.39 ± 0.07	1.53 ± 0.04
ATP-dependent function		
ATPase activity†	15–21	<2% of control
Phosphorylation by ATP*	1.6–2.2	<1% of control
ATP-displaceable eosin binding‡	11.8	<0.5
Inhibition of Rb occlusion§	70	Undetectable

*Units are nmol/mg of protein; occlusion data are mean ± SEM.
†Units are μmol per min per mg of protein.
‡Units are percent of total fluorescence.
§Units are percent inhibition at 0.1 mM Rb ± 1 mM ATP.

and the β chain are digested and a 13-kDa fragment was produced with loss of Rb occlusion. The results shown in Fig. 1 suggested that the 19-kDa fragment is essential for Rb occlusion. Fig. 2 tests this quantitatively. Trypsin was added to membranes containing the 19-kDa fragment, 1:50 (wt/wt), in a medium containing Rb and Ca ions. At various times from 10 sec to 4 min trypsin inhibitor was added. Rb occlusion was measured on portions of the suspension. Others were applied to 10–20% Mini-Gels. The absorbance at 595 nm of Coomassie blue extracted from the 19-kDa bands (proportional to protein concentration in this range) was measured. The correlation in Fig. 2 (slope, 1.06 ± 0.06) indicates that the 19-kDa fragment is essential for Rb occlusion. In this experiment, performed at lower trypsin/membrane protein ratio and shorter times than in Fig. 1, the β chain was not significantly digested although the 19-kDa fragment had disappeared (data not shown).

Are other peptides observed in Fig. 1 necessary for occlusion? An experiment examining tryptic digestion at various pH values showed that, as pH was lowered from 9.0 to 6.5, the β chain was largely digested to about 40 kDa and the 16-kDa fragment accumulated but the amount of the 19-kDa fragment and Rb occlusion were unchanged (data not shown). Thus an intact β chain is not necessary.

For sequencing from its N terminus, the 19-kDa fragment was transferred to a PVDF sheet. In two experiments, yields of N-terminal residues were sufficient to identify the first 16 residues as Asn-Pro-Lys-Thr-Asp-Lys-Leu-Val-Asn-Glu-Arg-Leu-Ile-Ser-Met-Ala.

This unambiguously identifies the N terminus of the 19-kDa fragment as residue 830 of the α chain (see Fig. 5).

Table 1 records the results of studies of ion occlusion and ATP-dependent functions in control and membranes containing the 19-kDa fragment. Note particularly that membranes containing the 19-kDa fragment occlude both Rb and Na. The specific activity for both Na and Rb occlusion is about twice that of control membranes, consistent with loss of about half the protein. The ratio of Na to Rb occlusion is not significantly different from 1.5 in both control and proteolyzed membranes. In a previous study (8), the stoichiometry of Na and Rb occlusion was shown to be three Na or two Rb per phosphoenzyme molecule. Thus all three Na-, or two Rb-, occluding sites are intact. [Not shown is that oubain inhibits Rb occlusion with a low affinity ($K_d \approx 400$ μM).]

Since the 19-kDa fragment lacks the major hydrophilic loop of the α chain, one might expect ATP-binding and ATP-dependent functions to have been lost. This is confirmed in Table 1. ATPase activity, phosphorylation by ATP, high-affinity binding of eosin, an ATP analogue (18), and also the inhibitory effect of ATP on Rb occlusion are undetectable.

Fig. 3 demonstrates interesting differences in cation occlusion, even though the maximal capacities of control and membranes containing the 19-kDa fragment are unaltered.

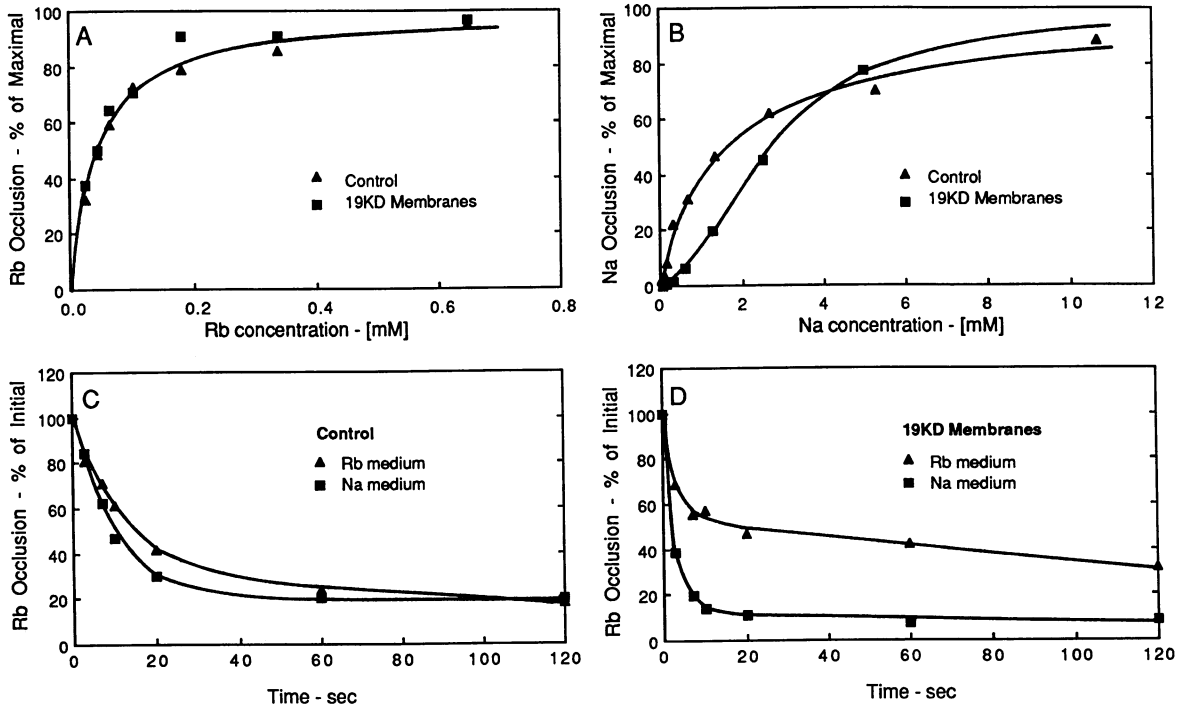

FIG. 3. Rb and Na affinity (*A* and *B*) and dissociation rates of ^{86}Rb into Rb- or Na-containing medium (*C* and *D*) from control and membranes containing the 19-kDa fragment. Theoretical curves for hyperbolae (*A*), Hill equation (*B*), or double-exponential decay (*C* and *D*), using the best-fit parameters quoted in the text, are superimposed on the experimental points (symbols as indicated).

Occlusion of Rb and Na was determined at suitable ion concentrations and maximum capacities determined by curve fitting. Fig. 3 *A* and *B* plots occlusion (as a fraction of maximal capacity) versus Rb and Na concentration, respectively. For Rb, control and membranes containing the 19-kDa fragment are practically indistinguishable, yielding simple hyperbolae with best-fit K_m values of 47 ± 3 and 38 ± 6 μM, respectively. In contrast, Na occlusion is essentially hyperbolic for control (K_m = 1.56 ± 0.30 mM; Hill number n = 0.92) (see also ref. 8) but is highly cooperative for membranes containing the 19-kDa fragment, with a somewhat lower apparent affinity ($K_{1/2}$ = 2.69 ± 0.13 mM; Hill number of n = 1.90).

Deocclusion of ^{86}Rb into medium containing unlabeled Rb or Na was measured. For control membranes (Fig. 3*C*) as reported (9), deocclusion is largely monophasic down to some 20% of the initial value, with little difference between Rb or Na medium (rate constants, 0.076 and 0.094 sec^{-1}, respectively). In contrast, for membranes containing the 19-kDa fragment (Fig. 3*D*), although deocclusion into Na is still largely monophasic (rate constant, 0.39 sec^{-1}), deocclusion into Rb is markedly biphasic, with rate constants of 0.36 and 0.0045 sec^{-1} and almost equal amplitudes of 45% and 55%, respectively. The strongly biphasic curve is reminiscent of a similar phenomenon reported for control enzyme in the presence of phosphate (19, 20). It is also observed at 0°C in the absence of phosphate (unpublished data).

An obvious question was whether the entire cation transport pathway was intact in the membranes containing the 19-kDa fragment. In reconstituted proteoliposomes, ATP-dependent Rb and Na fluxes should be absent but passive Rb fluxes in the absence of ATP and other ligands (21) could persist. After reconstitution, ^{86}Rb uptake was measured into Rb-loaded vesicles from medium containing a fixed amount of isotope and either a low (0.25 mM) or a high (20 mM) concentration of total RbCl (Fig. 4*A*). The difference in uptake at low and high Rb represents the saturable, that is pump-mediated, component. The flux at 20 mM RbCl represents passive leak. The saturable Rb uptake occurs in two phases; one is rapid and the other is far slower. The K_m for slow uptake is 0.48 mM (data not shown), similar to that for Rb–Rb exchange in control vesicles (21), but the rate is only 1–2% of that of control. The amount of Rb taken up in the rapid phase (at 1 mM Rb) is 5–7 pmol/10 μl of vesicles. This

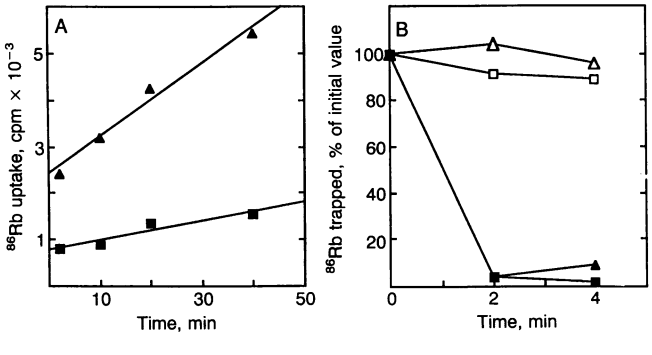

FIG. 4. Rb–Rb exchange in phospholipid vesicles reconstituted with membranes containing the 19-kDa fragment (*A*) and release of accumulated ^{86}Rb by valinomycin (*B*). (*A*) To calculate the quantity of Rb taken up in the initial phase and steady-state rate of ^{86}Rb flux, the slope and intercept of the regression lines at 20 mM Rb (■) were subtracted from those at 0.25 mM Rb (▲). (*B*) Rb-loaded vesicles (100 μl) were incubated for 2.5 min (triangles) or 10 min (squares) in the standard medium containing 1 mM Rb and ^{86}Rb (2.7×10^5 cpm). The vesicles with associated ^{86}Rb were eluted on a Dowex-50 column, and unlabeled 150 mM RbCl was added. The suspension was divided, 1 μM valinomycin (solid symbols) or ethanol (open symbols) was added, and after incubation at 20°C for 0, 2, or 4 min, samples were transferred to a second set of Dowex-50 columns, and vesicles were eluted.

is close to the pump density in vesicles containing control Na/K-ATPase [estimated from the phosphoenzyme (22)]. A similar biphasic time course was observed for control vesicles at 0°C (unpublished data). We propose (see *Discussion*) that the fast phase reflects one turnover of the proteolyzed enzyme, followed by a slow steady-state Rb–Rb exchange. This implies that ^{86}Rb is transported into the vesicles rather than being bound or occluded. To test this (Fig. 4*B*), vesicles were loaded with ^{86}Rb for 2.5 or 10 min, the external isotope was removed, and 150 mM RbCl was added. The amount of ^{86}Rb associated with vesicles was measured over 4 min, in the absence or presence of valinomycin. Clearly, with valinomycin, the ^{86}Rb associated with the vesicles both at 2.5 and 10 min is removed rapidly (by ^{86}Rb–Rb exchange). Thus the ^{86}Rb had been transported into the vesicles.

DISCUSSION

Structure. For purpose of discussion, Fig. 5 depicts a hypothetical model for the membranes containing the 19-kDa fragment, based on the arrangement of α and β chains suggested by Ovchinnikov *et al.* (5, 23), consistent with a finding that the C terminus is extracellular (24). In addition to the 19-kDa polypeptide and β chain, other membrane-spanning segments that cannot be further hydrolyzed by trypsin are assumed to be present. These fragments are shown as shaved to the tryptic limit. They might be somewhat longer. We also assume that the 19-kDa fragment extends to the C terminus. It might be slightly shorter.

Figs. 1 and 2 show that the 19-kDa fragment is essential for the Rb occlusion but, although highly suggestive, do not prove that the ion-binding residues reside on that segment. However, this is most probably the case. As mentioned above, studies with DCCD (8, 10) suggest that two carboxyl groups on the α chain participate in occlusion of Rb and Na. Recent experiments demonstrate that [^{14}C]DCCD labels the 19-kDa fragment strongly and potassium protects it (10). Two obvious candidates for potassium-binding carboxyls are Glu-953 and -954 (Fig. 5). Although the 19-kDa fragment is necessary, it is quite probable that the cation-occluding structure also comprises other transmembrane segments, the cation-binding cage consisting of oxygen-containing ligating groups donated by more than one segment. A major challenge will now be to identify other membrane-embedded tryptic fragments which are involved, and so to attempt to define a complete cation-occluding structure.

Gastric H,K-ATPase is inactivated by a light-sensitive derivative of the potassium-competitive inhibitor 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine (25). The N-terminal sequence Leu-Val-Asn-Glu of a photolabeled 7-kDa tryptic fragment (26) is in a location close to that of the N terminus of our 19-kDa fragment. Residues capable of binding potassium ions may be located in C-terminal segments of these homologous ATPases.

Function. ATP-binding and ATP-dependent functions have clearly been destroyed in membranes containing the 19-kDa fragment. Cation-binding functions seem largely intact, although interesting differences appear between control and membranes containing the 19-kDa fragment (it would be extraordinary if there were no differences). Membranes containing the 19-kDa fragment strongly display sigmoidal Na activation whereas control membranes are close to hyperbolic (Fig. 3*B*). Binding of three Na with hyperbolic activation implies that binding affinities of at least two sites are widely separated (see refs. 8 and 27). We suspect that the two carboxyl-containing sites, rather than the third neutral site, have the higher affinity. An economical explanation of the sigmoidal curve would be that the affinity of the higher affinity sites has been reduced. Apparent Rb affinity, on the other hand, seems largely unaffected (Fig. 3*A*). This seems

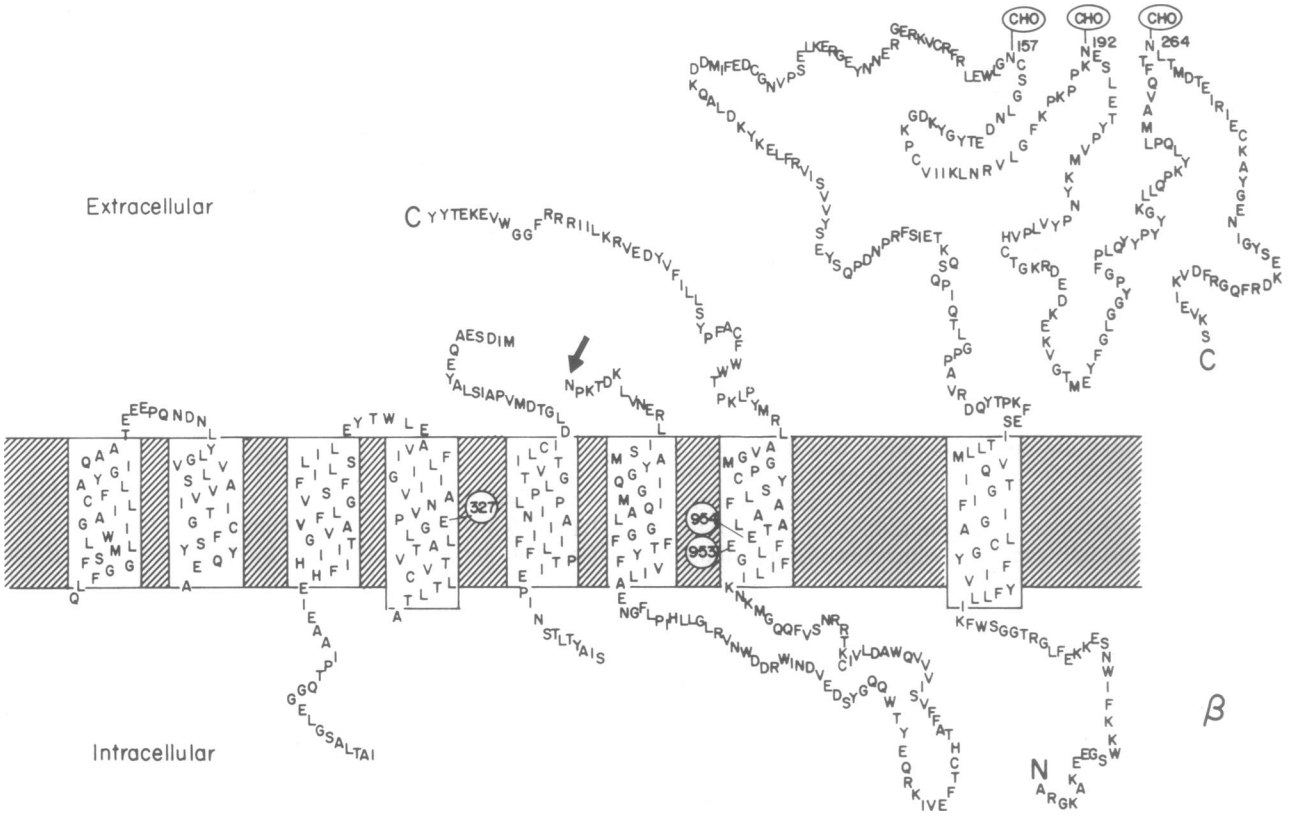

FIG. 5. Hypothetical arrangement of polypeptides in membranes containing the 19-kDa fragment.

paradoxical since two Rb and two of the Na ions are thought to bind to the same sites. However, apparent Rb and Na affinities are complex, reflecting both intrinsic binding and conformational changes that trap the cations (8, 16). Conformational changes involved in Rb occlusion or oligomycin-induced Na occlusion may be affected differently by proteolysis.

The major significance of Fig. 4 is the demonstration that the transport pathways for Rb in membranes containing the 19-kDa fragment remain accessible. The phenomena observed are understandable in terms of the evidence for biphasic release of occluded Rb shown in Fig. 3*D*. Here, the first Rb was released rapidly, but the second took minutes to dissociate. We interpret the intercept of saturable uptake in Fig. 4*A* to reflect entry of one Rb in a first turnover, while dissociation of the second into the vesicles, and hence the steady-state rate of Rb/Rb exchange, is greatly retarded by unlabeled Rb within the vesicles.

Implications. It seems clear that the cation and ATP binding sites are physically separate. ATP-binding residues reside on the large cytoplasmic loop (1, 2). The present findings and studies with DCCD (8, 10) imply that cation binding sites lie within transmembrane segments and that the K sites and two Na sites are the same. Inevitably, coupling between ATP hydrolysis and cation pumping must involve indirect interactions between the ATP and cation sites, mediated presumably by conformation changes ($E_1 - E_2$ transitions; see ref. 2). An important question is: How is the force transmitted between the ATP and cation sites? Conceivably the loop of the 19-kDa fragment predicted to lie within the cytoplasm (see Fig. 5) acts as a physical coupling device, for it is adjacent to the central cytoplasmic loop and is in a position to interact with the ATP-binding site.

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