Solubilization of a Complex of Tryptic Fragments of Na,K-ATPase Containing Occluded Rb Ions and Bound Ouabain†

Eran Or, Rivka Goldshleger, Daniel M. Tal, and Steven J. D. Karlish*

Biochemistry Department, Weizmann Institute of Science, Rehovot, 76100, Israel

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ABSTRACT: The nonionic detergent $C_{12}E_{10}$ (polyoxyethylene 10-laurylether) has been used to solubilize a complex of tryptic fragments of Na,K-ATPase containing occluded Rb ions and bound ouabain. The aim was to define which fragments are required to maintain Rb occlusion. The experiments utilize "19 kDa membranes" consisting of a 19 kDa and several smaller tryptic fragments (8−11.7 kDa) of the α subunit, which include trans-membrane segments M7/M10 and the pairs M1/M2, M3/M4, and M5/M6 [Capasso, J. M., et al. (1992) *J. Biol. Chem. 267*, 1150−1158]. The β subunit is partially split into a 16 kDa fragment and a glycosylated ≈50 kDa fragment. Cation occlusion and ouabain binding are intact. After preincubation of "19 kDa membranes" with Rb (5 mM) and then ouabain (10 mM), 90−100% of occluded Rb was solubilized by $C_{12}E_{10}$ at 0 °C. All fragments of the α and β subunits, and also the γ subunit, were cosolubilized by $C_{12}E_{10}$, and were observed to sediment together on a sucrose density gradient as a complex containing occluded Rb ions. The soluble complex consists of a monomer containing one copy of each fragment, as indicated by size-exclusion HPLC, as well as estimates of specific Rb occlusion (20.0 ± 1.2 nmol/mg of protein). In the absence of Rb ions and ouabain, the complex was unstable. Whereas the 19 kDa fragment (M7−M10) and β subunit remained associated, the smaller fragments, containing M5/M6 and M3/M4 and M1/M2, and the subunit dissociated. Observations on the thermal inactivation of Rb occlusion, and effects of pH and ionic strength, suggest that the soluble complex is stabilized by multiple interactions, both within the lipid bilayer and in hydrophilic domains (e.g., salt bridges).

An understanding of the mechanism of an active cation pump such as Na,K-ATPase[1] requires knowledge of the structure of the cation binding sites. Whereas the kinetics of cation occlusion by Na,K-ATPase and Ca-ATPase have been studied extensively [see Glynn and Karlish (1990)], structural knowledge lags far behind. In the absence of definitive molecular structure, a variety of techniques are being used to obtain information on this question. Proteolysis experiments and site-directed mutagenesis indicate that cation occlusion sites lie within the membrane domain (Karlish et al., 1990; Capasso et al., 1992; Lingrel & Kuntzweiler, 1994; Andersen & Vilsen, 1995). Therefore, it is necessary to have knowledge of the topology and spatial organization of trans-membrane segments. The α subunit of Na,K-ATPase, like that of H,K-ATPase, is thought to consist of 10 trans-membrane segments [for recent data, see Goldshleger et al. (1995) and Bamberg and Sachs (1994)]. In contrast with the trans-membrane topology, the organization of trans-membrane segments in the plane of the membrane is completely unknown. For both Na,K-ATPase and H,K-ATPase, interactions between α and β subunits play an important role in maintaining native structure and normal function (Geering, 1991; Lemas et al., 1994; Chow & Forte, 1995). Thus, an important consideration in the experimental determination of the organization of the trans-membrane segments of the α subunit is that this be defined in the context of the functional α−β heterodimer.

Study of the structure of the trans-membrane domain of renal Na,K-ATPase, using biochemical techniques, is facilitated by the availability of a simplified, well-defined preparation, known as "19 kDa membranes" (Karlish et al., 1990). "19 kDa membranes" are obtained by extensive tryptic digestion of Na,K-ATPase in the presence of Rb ions and absence of Ca ions. The treatment removes about half of the protein and leaves in the membrane well-defined fragments, consisting primarily of the trans membrane segments of Na,K-ATPase with their connecting external loops, while the cytoplasmic loops have been largely removed (Capasso et al., 1992). The preparation contains a 19 kDa C-terminal fragment (N-terminal Asn831, M7/M10) and several smaller peptides (8−11.7 kDa) derived from the α subunit containing pairs of trans-membrane segments (N-terminal Asp68, M1/M2; N-terminal Ile263, M3/M4; N-terminal Gln737, M5/M6). About half of the β subunit is cut into a 16 kDa fragment (N-terminal Ala5) and a glycosylated ≈50 kDa fragment (N-terminal Gly143). "19 kDa membranes" lack all ATP-dependent functions, but retain full Rb and Na occlusion capacity (Karlish et al., 1990) and also the high-affinity site for cardiac glycosides (Schwappach et al., 1994).

The question arises as to the organization of fragments in "19 kDa membranes". Consistent with the preservation of

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* Correspondence should be addressed to this author at the Biochemistry Department, Weizmann Institute of Science, Rehovot 76100, Israel. Fax: 972 8 9344118. E-mail: BCKARLIS@WEIZMANN.WEIZMANN.AC.IL.

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[1] Abbreviations: $C_{12}E_8$, n-dodecyl octaethylene glycol monoether; $C_{12}E_{10}$, polyoxyethylene 10-laurylether; DCCD, dicyclohexylcarbodiimide; Na,K-ATPase, (sodium plus potassium)-activated adenosinetriphosphatase (EC 3.6.1.37); PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidine difluoride); SDS, sodium dodecyl sulfate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MES, 4-morpholineethanesulfonic acid.

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the functional properties, electron microscope studies (Ning et al., 1993a) and saturation transfer ESR spectroscopy (Esmann et al., 1994) indicate that the organization of the membrane domain in "19 kDa membranes" is essentially similar to that of native Na,K-ATPase. A number of observations on thermal inactivation and proteolytic digestion of "19 kDa membranes" have led us to propose that the fragments interact as a cation-stabilized complex (Shainskaya & Karlish, 1994). At 37 °C, Rb occlusion is rapidly inactivated in the absence of occluded cations, while the presence of occluded cations (Rb, K, or Na) strongly protects against thermal inactivation (Or et al., 1993). Ouabain binding and Na binding associated with charge movement are thermally inactivated at the same rate, suggestive of disruption of the complex (Schwappach et al., 1994). Proteolysis experiments provide more detailed information (Shainskaya & Karlish, 1994). When occluded Rb ions are displaced by Ca, Rb occlusion is thermally inactivated, and then all the fragments in "19 kDa membranes" are digested further by trypsin. Analysis of the remaining fragments showed that displacement of occluded Rb ions exposed extramembrane loops and tails of all tryptic fragments of the α, β, and γ subunits to further digestion. Conversely, it could be concluded that occluded Rb ions induce stabilizing interactions which protect all fragments of α and β subunits against further digestion.

The object of the present work has been to obtain direct evidence for a functional complex of tryptic fragments, by solubilizing "19 kDa membranes" with a detergent in a way which preserves Rb occlusion. Previously, the mild nonionic detergent $C_{12}E_8$ has been used extensively to solubilize Na,K-ATPase in a functional state (Craig, 1982; Brotherus et al., 1983; Hayashi et al., 1983, 1989; Esman & Skou, 1984). Occlusion of Rb or Na ions in native Na,K-ATPase solubilized with $C_{12}E_8$ has been described for both shark rectal gland (Esmann, 1985) and renal enzyme (Vilsen et al., 1987). In the latter case, optimal preservation of solubilized occlusion activity was found to depend on the presence of the occluded Rb or Na ions during solubilization. Esmann and Skou (1984) reported that whereas $C_{12}E_8$, at high concentrations, inactivates the solubilized enzyme, $C_{12}E_{10}$ is much less deleterious. These authors suggested that $C_{12}E_{10}$ is a better substitute for the displaced lipids, because it is a polydisperse detergent with a range of chain lengths, while $C_{12}E_8$ is a chemically pure compound with a single chain length. Therefore, we have chosen to solubilize "19 kDa membranes" with $C_{12}E_{10}$, and look for Rb occlusion activity released into the supernatant. We describe here conditions for solubilization of "19 kDa membranes" with preservation of Rb occlusion, the peptide composition of a soluble complex, and factors affecting stability of the complex.

MATERIALS AND METHODS

Na,K-ATPase was prepared from pig kidney red outer medulla according to the rapid procedure of Jørgensen (1974) and stored at −70 °C. Protein concentration and ATPase activity were determined as described by Jørgensen (1974). Specific activities were 13−18 units/mg of protein. Before use, enzyme was thawed and dialyzed overnight at 4 °C against 1000 volumes of 25 mM histidine, 1 mM EDTA (Tris), pH 7.0.

Tryptic Digestion of Na,K-ATPase. "19 kDa membranes" were prepared as described by Capasso et al. (1992), and resuspended at 3 mg/mL in 2 mM RbCl, 25 mM imidazole, and 1 mM EDTA, pH 7.5.

Solubilization of "19 kDa Membranes". "19 kDa membranes" were incubated with 8.5 mM choline chloride, 5 mM RbCl + ^{86}Rb [$(0.8-1.2)\times10^5$ cpm/μL] for 20 min at 22 °C. Ouabain (final concentration 10 mM) was then added together with 8.5 mM choline chloride, 5 mM RbCl + ^{86}Rb of the same specific activity. After 40 min, the mixture (0.8 mg of protein/mL) was mixed on ice with an equal volume of ice-cold solution containing 10 mM ouabain, 8.5 mM choline chloride, 5 mM RbCl + ^{86}Rb, and $C_{12}E_{10}$ at the concentration required to produce the desired final weight: weight ratio of detergent to protein. In order to minimize inactivation of "19 kDa membranes" during solubilization, the detergent solution was added with manual mixing in three aliquots. The solubilized mixture was centrifuged at 100000*g* or at 250000*g* for 1 h at 4 °C, and the supernatant was collected and kept on ice. The pellet, unless indicated otherwise, was discarded. When solubilized "19 kDa membranes" were to be resolved by SDS−PAGE, tracer Rb was omitted.

Rb Occlusion Assays. These were performed according to the method of Shani et al. (1987). Usually 50 μL samples of solubilized "19 kDa membranes" (≈7 μg) containing 5 mM RbCl + $(4-6)\times10^6$ cpm ^{86}Rb were transferred to Dowex columns and eluted with 1.5 mL of ice-cold 0.2 M sucrose.

Sucrose Density Gradient Centrifugation. A linear gradient of 11mL of 5−25% sucrose in 3.1 mM imidazole, 125 μM EDTA, pH 7.5, 5 mM RbCl, 10 mM ouabain, and 0.2 mg/mL $C_{12}E_{10}$ was prepared, and the tubes were stored for several hours at 4 °C before use. "19 kDa membranes" were solubilized at a detergent to protein ratio of 2.5:1 w/w, and 1 mL (∼130 μg of protein) was layered gently on top of the gradient. Tubes were then centrifuged in an SW41 rotor at 274000*g* for 16 h at 4 °C. The gradient was displaced from the bottom with a 40% sucrose solution, and aliquots were collected from the top through a homemade funnel.

Size-Exclusion HPLC. The HPLC system consists of a Rheodyne 5092 injector, a Waters 600E pump, and a Waters 991 photodiode array detector operating at wavelengths between 227 and 351 nm, at a resolution of 2 nm, and a sampling time of 200 ms taken at intervals of 5 s. The system is controlled by an NEC computer equipped with acquisition and processing software. A column of TSK-g3000SW (7.5 mm internal diameter × 60 cm length) was connected in series with a TSK guard column SW (7.5 mm internal diameter ×7.5 cm) and used for the separation, with the appropriate buffer (see figure legends), in the isocratic mode. The flow rate was 0.5 mL/min.

Gel Electrophoresis. The Tricine−SDS−PAGE system of Schägger and von Jagow (1987) was used. Either 10% or 16.5% 1 mm gels (23 cm long) were run according to the modifications of Capasso et al. (1992). Intact "19 kDa membranes" were first dissolved with 2% SDS, before delipidation. All protein samples were delipidated with 4 volumes of an ice-cold mixture of methanol/ether (2:1 v/v) and stored overnight at −20 °C. Precipitated protein was collected by centrifugation at 9700*g* for 1 h, and dried under

a stream of nitrogen. The dry pellets were dissolved in 70 μL of gel sample buffer.

Transfer to PVDF Paper and Peptide Sequencing. For sequencing, stained bands were cut out of a 16.5% gel and rerun on a 1.5 mm 10% Tricine gel (11.5 cm long), and peptides were electroblotted onto PVDF paper according to Matsudaira (1987), as described by Capasso et al. (1992). Sequencing was done on an Applied Biosystems Model 475A protein sequencer with an on-line Model 120A phenylthiohydantoin analyzer. Yields were 10−27 pmol of amino acids per cycle.

Preparation of Rabbit Antiserum against the γ Subunit. Renal Na,K-ATPase (4−5 mg) was solubilized with 2% SDS and was delipidated with 4 volumes of methanol as described above. The dry pellet was dissolved in sample buffer and separated on a 10% gel. The two bands of the γ subunit were cut out of the stained gel, homogenized in 3 mL of 2% SDS, 10 mM Tris-HCl, pH 8.0, and vortexed at room temperature for several hours. The homogenate was then centrifuged at 9700*g* for 30 min, and the supernatant was set aside. The pellet was extracted again in the same buffer overnight. Both supernatants were combined and concentrated with Centricon X10, and the retentates were precipitated with 4 volumes of ice-cold acetone plus 7 mM acetic acid and stored at −20 °C for several days. The precipitate was collected by centrifugation at 9700*g* for 1 h, dried, and dissolved in 150 μL of 0.5% SDS (∼200 μg). The protein was used to prepare rabbit antiserum against the γ subunit according to the procedure of Grossman et al. (1990).

Immunoblotting. Peptides were resolved on a 10% gel, electroblotted onto PVDF paper, and reacted with γ antiserum (diluted 1:160) as described in Capasso et al. (1992). Immunoblots were stained with diaminobenzidine with metal ion enhancement (Harlow & Lane, 1988).

Calculations. Best-fit parameters were calculated by nonlinear regression using the program Enzfitter (Elsevier-BIOSOFT) for IBM-PC.

Materials. ^{86}RbCl was obtained from Du Pont NEN. Dowex 50W-X8 100 mesh, H-form, was obtained from Sigma and converted to Tris-form before use. Choline chloride was purchased from Fluka and was recrystallized from hot ethanol. TPCK-trypsin (bovine pancreas) was from Worthington. Trypsin inhibitor (type 1-S from soybean), ouabain, thioglycolate, Tricine, $C_{12}E_{10}$, and molecular mass markers (2.5−16.9 kDa) were purchased from Sigma. Electrophoresis grade reagents for SDS−PAGE were obtained from Bio-Rad. PVDF paper was from Millipore. Centricon concentrators were obtained from Amicon.

RESULTS

Solubilization of Occluded Rb Ions in the Presence of Ouabain. Figures 1 and 2 and Tables 1 and 2 demonstrate the basic features of solubilization of "19 kDa membranes" by the nonionic detergent $C_{12}E_{10}$, with retention of Rb occlusion. We define as "soluble", the material remaining in the supernatant following centrifugation for 1 h at 100000*g* or higher forces.

Preliminary experiments showed that the ability to occlude Rb ions was not preserved upon solubilizing "19 kDa membranes" in the absence of Rb ions, and was only partially preserved upon solubilization in the presence of Rb ions (see Table 1). Ouabain has been shown to greatly decrease dissociation of occluded Rb from native Na,K-ATPase (Forbush, 1983). Thus, it seemed possible that the presence of ouabain might help to stabilize Rb occlusion of solubilized

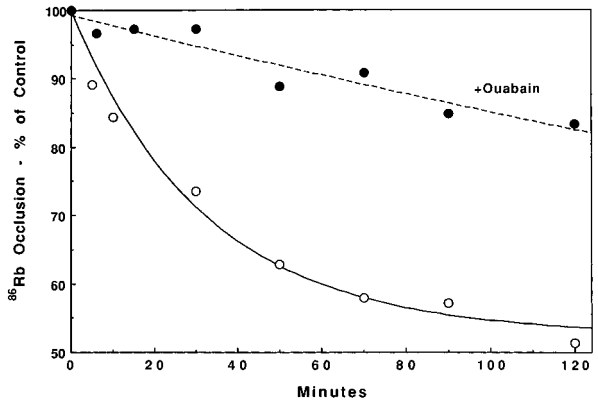

FIGURE 1: Effect of ouabain on dissociation of ^{86}Rb from "19 kDa membranes". "19 kDa membranes" were suspended at 0.4 mg/mL in a medium containing 23 mM imidazole, 0.9 mM EDTA, pH 7.5, and 5 mM RbCl + ^{86}Rb (10^5cpm/μL) in the absence of ouabain (○), or ouabain (10 mM) was added after 20 min and the suspension was incubated at 20 °C for a further 40 min (●). 50 μL aliquots of the suspensions were mixed at 0 °C with 50 μL of 0.1 M RbCl, and after different time intervals, samples were transferred to Dowex columns. Control (t_0) was taken as the Rb occlusion 20 s after intiation of dissociation. Dissociation of Rb without ouabain was fitted with the equation $Y = A\exp(-kt) + (100 - A)$ where $A = 47.5 \pm 2.3\%$ and $k = 0.031 \pm 0.005$ min^{-1}. Dissociation of Rb in the presence of 10 mM ouabain was fitted with the equation $Y = A\exp(-kt)$ where $A = 99.2 \pm 1.2\%$ and $k = 0.0015 \pm 0.0002$ min^{-1}.

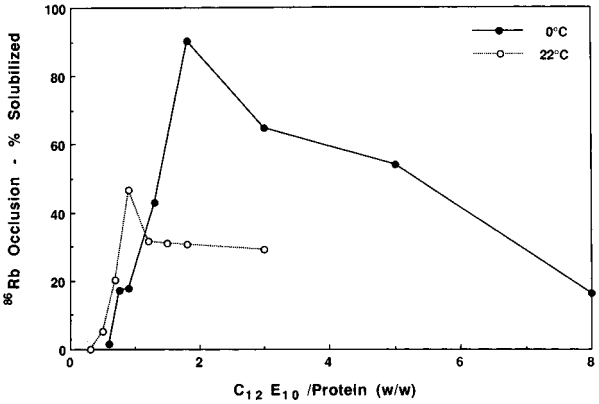

FIGURE 2: Solubilization of the Rb occlusion activity of "19 kDa membranes" at increasing ratios of $C_{12}E_{10}$ to protein. "19 kDa membranes" were solubilized with increasing ratios of $C_{12}E_{10}$ to protein (0.6−8 w/w) as described under Materials and Methods. Two 50 μL aliquots of the supernatant of each ratio were assayed for Rb occlusion activity, which is presented as a percent of the occlusion activity of an identical but unsolubilized sample. (●) Solubilization at 0 °C. (○) Solubilization at 22 °C.

Table 1: Effect of Rb Ions and Ouabain on the Yield of Solubilized Rb Occlusion Activity[a]

	Rb occlusion (% of control)	
[Rb] (mM)	−ouabain	+7 mM ouabain
1.0	22.2	64.8
2.5	31.3	93.1
5.0	46.9	101.4

[a] "19 kDa membranes" were solubilized with $C_{12}E_{10}$ (ratio 2:1 w/w) in the presence of either 1 mM, 2.5 mM, or 5 mM RbCl (+^{86}Rb) without or with 7 mM ouabain. Rb occlusion in the supernatants was determined and expressed as a percent of the Rb occlusion ([Rb] = 1 mM) activity of unsolubilized "19 kDa membranes".

Table 2: Controls for the Specificity of the Solubilized Rb Occlusion Activity[a]

treatment	Rb occlusion (% of control)
none	91.6
DCCD	30.9
trypsinized	11.0
SDS	4.0

[a] DCCD: "19 kDa membranes" (100 μL, 1.2 mg/mL) suspended in 22 mM imidazole, 0.9 mM EDTA, pH 7.5, were incubated for 1 h at 22 °C either in the absence (none) or in the presence of 160 nmol of DCCD. Both samples were then centrifuged for 15 min at 30 psi in a Beckman airfuge; pellets were washed and resuspended at 3 mg/mL in 25 mM imidazole, 1 mM EDTA, pH 7.5. Trypsinized: "19 kDa membranes" (180 μL, 830 μg/mL) in 22 mM imidazole, 0.9 mM EDTA, pH 7.5, 550 μM RbCl, and 3 mM $CaCl_2$ were treated overnight with trypsin (10% w/w) at 37 °C. Membranes were then centrifuged for 15 min at 30 psi in a Beckman airfuge; the pellet was washed and resuspended at 3 mg/mL in 25 mM imidazole, 1mM EDTA, pH 7.5. 19 kDa membranes, subjected to the above treatments, were incubated with 3 mM RbCl + ^{86}Rb, ouabain was then added to 7 mM, and membranes were solubilized with $C_{12}E_{10}$ at 2.5 (w/w). Supernatants were assayed for Rb occlusion. SDS: $C_{12}E_{10}$-solubilized "19 kDa membranes" (5 mM RbCl + ^{86}Rb plus 7 mM ouabain) were mixed with SDS at 1% and then assayed for Rb occlusion activity. Data are presented as a percent of the Rb occlusion capacity of unsolubilized "19 kDa membranes".

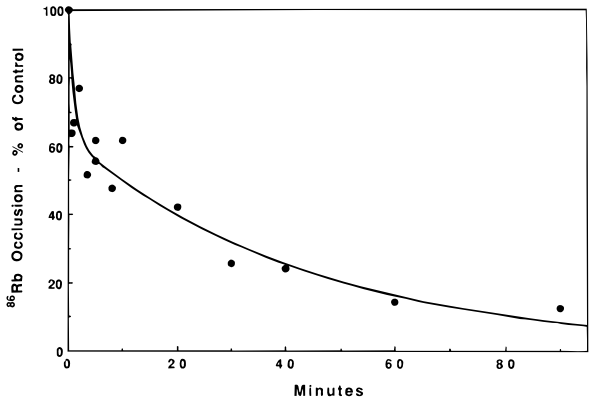

FIGURE 3: Dissociation of ^{86}Rb from solubilized "19 kDa membranes". "19 kDa membranes", preincubated with 3 mM RbCl + ^{86}Rb (10^5 cpm/μL) plus 5 mM ouabain, were solubilized with $C_{12}E_{10}$ at a ratio of 1.92 w/w as described under Materials and Methods. The ionic strength of the supernatant was adjusted to 30 mM with 650 mM choline chloride. 50 μL portions of the supernatant were mixed at 15 °C with 100 μL of 30 mM RbCl, 5 mM ouabain. After different time intervals, samples were transferred to Dowex columns. Data were fitted with the equation $Y = 50\ \exp(-k_1 t) + 50\ \exp(-k_2 t)$ where $k_1 = 0.56 \pm 0.19$ min^{-1} and $k_2 = 0.0155 \pm 0.0041$ min^{-1}.

"19 kDa membranes". In view of the well-known ouabain−K antagonism (Glynn, 1957), it could be expected that a high concentration of ouabain would be required. As a first step, we examined the effect of ouabain on deocclusion of ^{86}Rb from "19 kDa membranes" into a medium containing an excess of nonradioactive Rb (Figure 1). At 0 °C, 50% of the occluded Rb dissociated within 2 h (rate constant 0.03 min^{-1}). The presence of 10 mM ouabain in the medium slowed the dissociation by 20-fold, only 17% of the occluded Rb being released during 2 h (rate constant 0.0015 min^{-1}). The apparent affinity for ouabain for this effect was 1−2 mM. Despite the high concentration of ouabain used, the large reduction in the dissociation rate of occluded Rb argues for a specific effect of ouabain in these conditions. The experiment demonstrates the existence of a ternary complex between ouabain, occluded Rb ions, and "19 kDa membranes".

"19 kDa membranes" were preequilibrated with 5 mM Rb ions (+^{86}Rb) and then with 10 mM ouabain and were then solubilized at increasing detergent:protein ratios, at 0 and 22 °C (Figure 2). At 0 °C, Rb occlusion capacity was progressively solubilized at ratios of detergent to protein above 0.7 (w/w), reaching ≈90% solubilization at the detergent to protein ratio of 1.8 (w/w). At detergent to protein ratios above 2 (w/w), the solubilized Rb occlusion capacity was progressively inactivated, dropping to 16% of control at a ratio of 8 (w/w). Solubilization of Rb occlusion capacity at 22 °C occurred over a lower and narrower range of detergent to protein ratios (0.5−0.9 w/w), but no more than 46% could be solubilized.

The experiment documented in Table 1 compared the yield of Rb occlusion after solubilizing "19 kDa membranes" preincubated with three concentrations of Rb (1, 2.5, and 5 mM) in the absence and presence of ouabain (7 mM). Rb occlusion was solubilized less efficiently in the absence of ouabain, although the yield was improved from 22.2% to 46.9% upon raising the Rb concentration from 1 to 5 mM. Preincubation with ouabain (7 mM) prior to solubilization improved the yield 2−3-fold, and led to essentially quantitative solubilization of Rb occlusion capacity. For subsequent experiments, 5 mM Rb ions and 10 mM ouabain were used.

The necessity for addition of ouabain in order to fully solubilize Rb occlusion provides one indication that the occlusion is specific, and does not represent nonspecific binding of Rb ions to solubilized protein or lipid. Table 2 presents results of a number of additional control experiments which strengthen that conclusion. Incubation of "19 kDa membranes" with DCCD (Goldshleger et al., 1992) or digestion of "19 kDa membranes" by trypsin in the presence of Ca ions (Shainskaya & Karlish, 1994) or SDS reduced the solubilized Rb occlusion capacity to 31%, 11%, and 4% of control levels, respectively. These treatments are known to inactivate Rb occlusion in intact "19 kDa membranes". In addition, preincubation of "19 kDa membranes" with ouabain plus Mg ions partially reduces the yield of the solubilized Rb occlusion activity (data not shown).

Dissociation of ^{86}Rb from intact "19 kDa membranes" into a Rb-containing medium is biphasic (Karlish et al., 1990), a phenomenon thought to reflect ordered dissociation of the two occluded Rb ions (Glynn et al., 1985; Forbush, 1987). It was of interest to establish whether this feature is preserved in solubilized "19 kDa membranes". Thus, we have looked at the rate of dissociation of ^{86}Rb from solubilized "19 kDa membranes" into a medium containing a 20-fold excess of nonradioactive Rb ions (Figure 3). Dissociation of ^{86}Rb indeed displayed biphasic kinetics with fitted rate constants of 0.56 min^{-1} and 0.015 min^{-1}, and equal amplitudes.

The specific Rb occlusion activity of solubilized "19 kDa membranes" was estimated and compared to that of intact "19 kDa membranes" by determining the Rb occlusion capacity and protein content of solubilized "19 kDa membranes". The specific Rb occlusion activity of intact "19 kDa membranes" was 6.16 ± 0.25 nmol of Rb/mg of protein ($n = 7$). At the optimal ratio of detergent to protein, 32.2 ± 1.4% ($n = 3$) of the protein in intact "19 kDa membranes" was solubilized as determined by Lowry protein assays. This relatively low percentage may be explained by the fact that the "19 kDa membranes" contain contaminating proteins

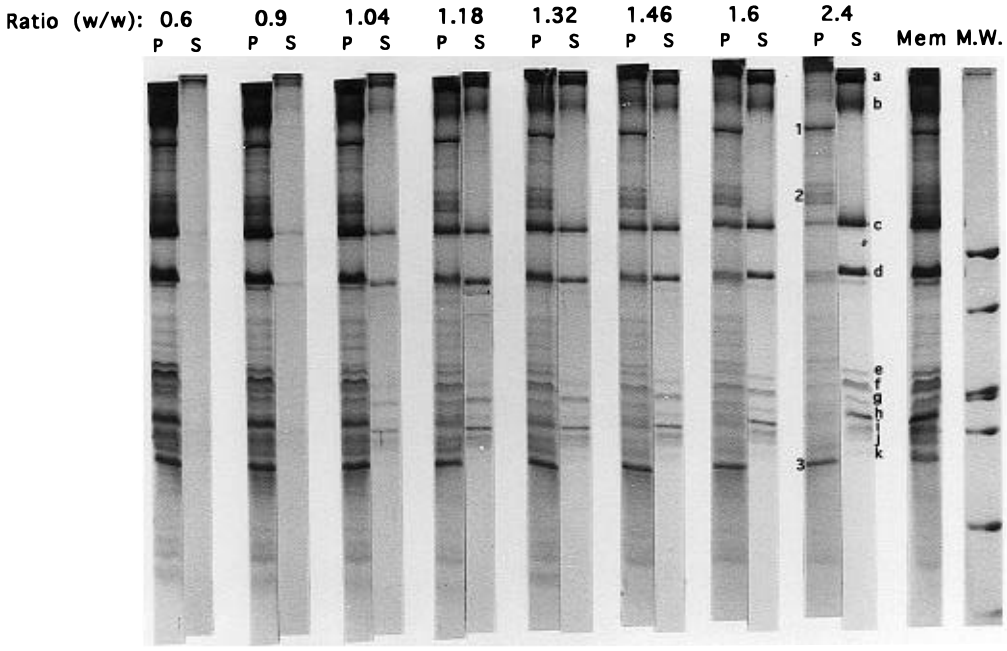

FIGURE 4: Solubilization of peptides of "19 kDa membranes". "19 kDa membranes" (~150 μg) were incubated with 5 mM Rb and 10 mM ouabain, solubilized with $C_{12}E_{10}$ at the indicated ratios of detergent to protein (w/w), and centrifuged (see Materials and Methods). Pellets were dissolved in 200 μL of 2% SDS. The concentration of detergent in each of the supernatants was adjusted to 0.96 mg/mL in order to ensure a uniform efficiency of precipitation. Samples were delipidated, dissolved in gel buffer, and resolved on a 16.5% gel. P, pellet; S, supernatant; Mem, unsolubilized "19 kDa membranes"; M.W., molecular mass standards (kDa): 16.9, 14.2, 10.7, 8.2, 6.2; (*a*) β subunit; (*b*) ≈50 kDa fragment; (*c*) 19 kDa fragment; (*d*) 16 kDa fragment; (*e*−*k*) peptides of 11.7−8 kDa; '1', 27 kDa fragment; '2', 23−21 kDa fragments; '3', 7.6 kDa fragment.

since they are prepared from Na,K-ATPase which is only about 35−50% pure. In addition, perhaps slightly less than 100% of the fragments in "19 kDa membranes" are solubilized at the optimal ratio of detergent to protein. Since 90−100% of the Rb occlusion capacity was solubilized, the average specific activity for the soluble preparation was calculated to be 18.2 ± 1.1 nmol of Rb/mg of protein. Previously, it was found that the Lowry assay overestimates the protein in the α subunit and 19 kDa fragment by 1.1-fold, by comparison with absolute amino acid analyses (Goldshleger et al., 1992). It is reasonable to assume that the same correction factor applies to the components of the soluble "19 kDa membranes", and thus the corrected specific activity would be 20.0 ± 1.2 nmol of Rb/mg of protein (see Discussion).

Peptide Components of a Soluble Complex. The peptide components of "19 kDa membranes" include the 19 kDa and five smaller fragments of 8−11.7 kDa, derived from the α subunit, the β subunit, which is partially cut into two fragments of 16 and ≈50 kDa, and presumably also the γ subunit, which runs as a doublet with masses of 10.9 and 9.5 kDa (Capasso et al., 1992; Mercer et al., 1993). Figure 4 presents an experiment designed to identify fragments specifically associated with the solubilized Rb occlusion activity. "19 kDa membranes" were solubilized with $C_{12}E_{10}$ at 0 °C at increasing detergent to protein ratios (0.6−2.4 w/w), the range over which Rb occlusion activity is solubilized. For each ratio of detergent to protein, the soluble protein fraction (S) and insoluble protein pellet (P) were applied to a 16.5% Tricine gel. At the lowest ratio (0.6 w/w), almost no protein was found in the soluble fraction while essentially all the protein remained in the pellet. As the ratio of detergent to protein was increased, a number of fragments were cosolubilized. The intensities of these fragments in the supernatant increased in parallel, as they were depleted from the pellet. The cosolubilized fragments include the intact β subunit (*a*), fragments of ≈50 kDa (*b*), 19 kDa (*c*), and 16 kDa (*d*); and seven peptides of 11.7−8 kDa (*e*−*k*). At the highest ratio of detergent to protein (2.4 w/w), these fragments were almost completely solubilized, and only a small proportion remained in the pellet (although there was some inevitable loss of protein, compared to the unsolubilized "19 kDa-membrane" sample). A number of contaminating proteins such as a 27 kDa fragment, three fragments of 23−21 kDa, and a 7.6 kDa peptide (Figure 4, fragments 1−3, respectively) were not solubilized and remained in the pellet even at the highest ratio of detergent to protein. The 7.6 kDa fragment has the N-terminal sequence DLGGSAP-LAIGFS (Capasso et al., 1992), and is derived from a water channel (Preston & Agre, 1991). No trace of this fragment was found in the soluble fraction. The level of background Coomassie staining of the gel was clearly reduced in the solubilized preparation, suggesting also that irrelevant proteins had been removed. Thus, solubilization of "19 kDa membranes" substantially purified the preparation from unrelated contaminant proteins. A similar observation was described previously for solubilization of native Na,K-ATPase by $C_{12}E_8$ (Esmann & Skou, 1984).

A likely explanation of cosolubilization of fragments seen in Figure 4 is that the solubilized fragments of "19 kDa membranes" form a complex. This hypothesis was tested by sedimenting the solubilized preparation on a linear sucrose density gradient (5−25%), in order to separate macromolecular complexes according to size (Figure 5). Centrifugation was done at 4 °C for 16 h,[2] and then each fraction from the gradient was assayed for Rb occlusion capacity and protein content (Figure 5A). The assay revealed one peak

[2] At 0 °C, the half-lifetime of Rb occlusion of the soluble complex is about 15 h (result not shown).

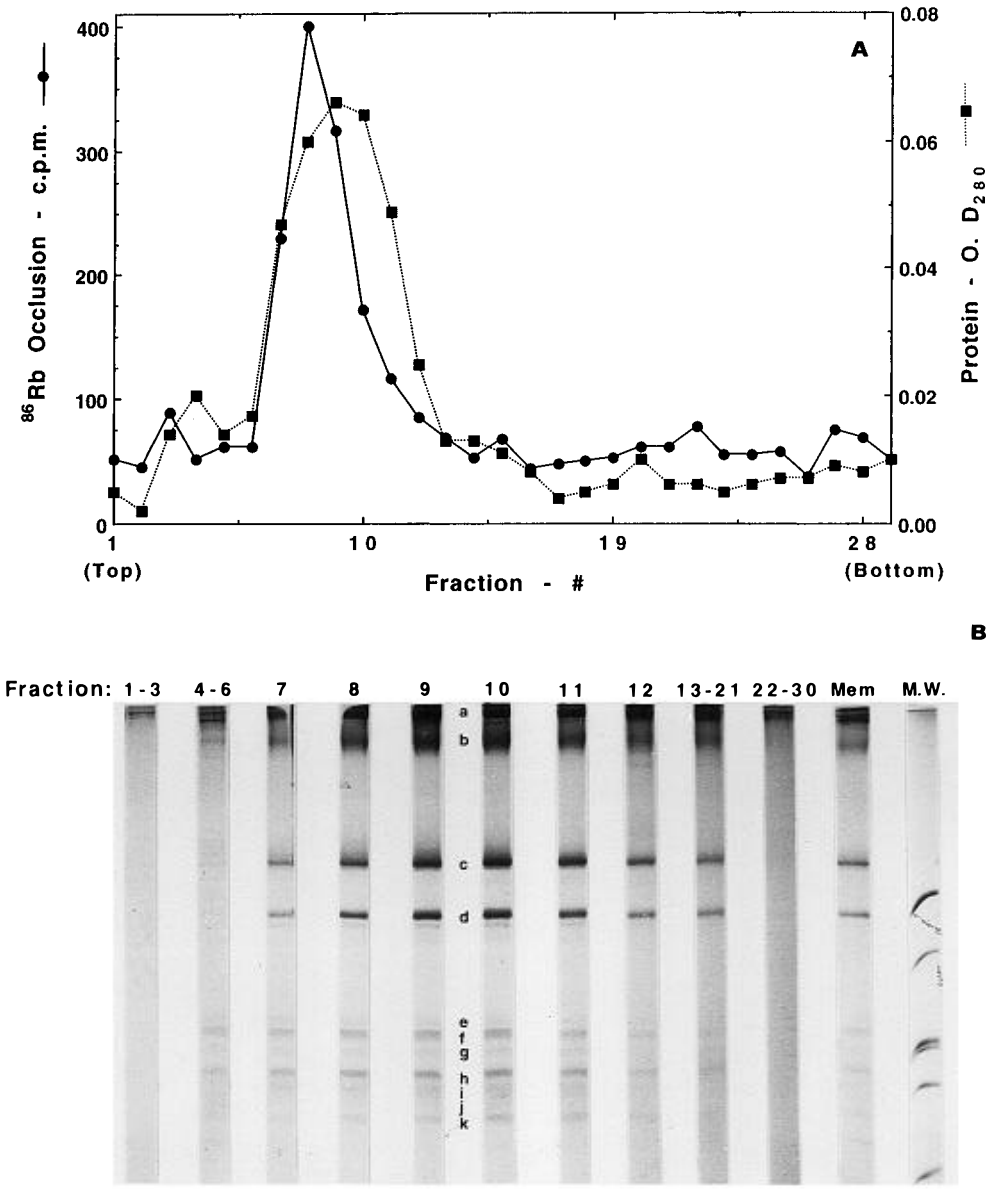

FIGURE 5: Sucrose density gradient profile of solubilized "19 kDa membranes". Details of centrifugation of solubilized "19 kDa membranes" on a linear 5−25% sucrose gradient are given under Materials and Methods. (A) 400 μL fractions were collected and assayed for Rb occlusion (closed circles) and for protein concentration (closed squares). For measurement of Rb occlusion, 45 μL of each fraction was mixed at 20 °C with 5 μL of 5 mM RbCl + ^{86}Rb (5.4×10^6cpm), and after 30 min, Rb occlusion was determined. The remainder of each fraction was used for determination of protein concentration (absorbance at 280 nm), and then it was delipidated, dissolved in sample buffer, and resolved on a 16.5% Tricine gel (B). The figure indicates which fractions were pooled together in each lane. Mem, solubilized "19 kDa membranes"; M.W., molecular mass markers (kDa): 16.9, 14.2, 10.7, 8.2, 6.2; (*a*) β subunit; (*b*) ≈50 kDa fragment; (*c*) 19 kDa fragment; (*d*) 16 kDa fragment; (*e*−*k*) peptides of 11.7−8 kDa.

of Rb occlusion activity (fractions 7−11), which overlapped the peak of protein (fractions 7−12). The protein peak extends one fraction to the right of the peak of occlusion, possibly due to the presence of protein aggregates, which are known to form during extended periods of storage [see Brotherus et al. (1983)]. The gel in Figure 5B shows the pattern of fragments in the fractions collected from the sucrose gradient. The fragments in "19 kDa membranes" which are cosolubilized (Figure 4, fragments *a*−*k*) are found predominantly in the same fractions of the sucrose density gradient where the Rb occlusion activity is found (Figure 5B, fractions 7−12). Inspection of these fractions shows a qualitative correlation in the intensity profiles of the ≈50 kDa (*b*), 19 kDa (*c*), and 16 kDa (*d*) fragments and three smaller peptides (*f*, *h*, *k*). Fractions 4−6 appear to contain some smaller peptides (8−11.7 kDa), but the amounts represent only a small proportion of their total. Previously, fragments *e*, *f*, and *g* were identified as those containing M1/M2 (N-terminal Asp[68]), fragment *i* contains M3/M4 (N-terminal Ile[263]), and fragment *j* contains M5/M6 (N-terminal Gln[737]) (Capasso et al., 1992).

Peptide *h* could not be sequenced, presumably because its N-terminus is blocked. Peptides *g*[3] and *h* were suggested previously to represent the γ subunit (Capasso et al., 1992) which runs as a doublet (Mercer et al., 1993). As seen in the immunoblot in Figure 6, peptides *g* and *h* are recognized by an antiserum prepared against the porcine γ subunit, a doublet being observed in both intact (Mem) and solubilized

[3] Fragment *g* contains two peptides; one of them is the γ subunit, and the other is a tryptic fragment of the α subunit containing transmembrane segments M1 and M2 (Capasso et al., 1992).

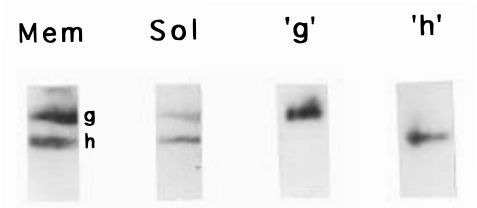

FIGURE 6: Immunoblot of "19 kDa membranes" using antiserum raised against the γ subunit. "19 kDa membranes" (Mem), solubilized "19 kDa membranes" (Sol), and fragments *g* and *h* of solubilized "19 kDa membranes", excised from a 16.5% Tricine gel, were all resolved on a 10 cm long 10% Tricine gel. The peptides were then electroblotted onto PVDF paper which was used for the immunoblot with rabbit antiserum raised against the γ subunit (see Materials and Methods).

(Sol) "19 kDa membranes". Unambiguous identification of fragments *g* and *h* as the γ subunit pair was made by the demonstration that both fragments displayed the expected electrophoretic mobility and recognized the anti-γ antibody when cut out of a 16.5% gel and rerun on a 10% gel (Figure 6, *g* and *h*). Fragment *k* was transferred to PVDF paper and was sequenced. The first seven residues were determined to be GDVDPFY. This fragment, which was also observed previously (Shainskaya & Karlish, 1994; Goldshleger et al., 1995), has 71% homology with residues 6−12 of the bovine γ subunit (Mercer et al., 1993); i.e., it appears to be a tryptic fragment of the porcine γ subunit.

In summary, it appears that all the peptides which are cosolubilized, and sediment together on the sucrose gradient, are derived from the α, β, or γ subunits of the pump.

Since the presence of Rb ions and ouabain is necessary for maintenance of Rb occlusion in solubilized "19 kDa membranes", it was of interest to examine whether the structural integrity of the solubilized complex of fragments depends also on the presence of Rb ions and ouabain. "19 kDa membranes" were solubilized with $C_{12}E_{10}$, in the absence of Rb ions and ouabain, and the supernatant was sedimented on the sucrose density gradient (5−25%). The protein profile in Figure 7A shows a broad peak (fractions 5−13) and a small shoulder (fractions 3−5). The peptide components of the fractions were analyzed in the gel shown in Figure 7B. It is striking that, whereas the β subunit (*a*) and the ≈50 kDa (*b*), 19 kDa (*c*), and 16 kDa (*d*) fragments were found together mainly in fractions 7−10, the smaller peptides (*e*−*k*) were displaced to the left. Peptides *e*−*h* and *k* were found mainly in fractions 6−9, while peptides *i* and *j* were displaced even further to the left, being found also in fractions 4 and 5, where other small peptides were negligible. Peptides *i* and *j* contain the trans-membrane pairs M3/M4 and M5/M6, respectively (see Discussion). Size-exclusion HPLC using a TSK-3000 column was also used to characterize the complex of tryptic fragments (Figure 8). Two peaks of protein were observed. The major peak (82% by area) contained all fragments of the α, β, and γ subunits in the complex, while the minor peak (18% by area) contained mainly the smaller 8−11.7 kDa fragments (gels not shown). An approximate size of the major complex could be estimated by comparing its elution time with those of soluble protein markers or those of separated α and β subunits of the pump. The complex eluted slightly after the α subunit and at the same position as a soluble marker of 128 kDa. This experiment indicates that the complex consists of a monomer containing one copy of each fragment (see Discussion).

Factors Affecting Stability of Rb Occlusion. We have found that, in addition to Rb ions plus ouabain, temperature, pH, and ionic strength substantially affect the stability of Rb occlusion of the soluble preparation.

In the experiment in Figure 9, the solubilized preparation was incubated at temperatures from 20 to 40 °C, and aliquots were withdrawn at various times to determine the remaining Rb occlusion capacity. The kinetics of loss of Rb occlusion were not described by a simple exponential decay, but could be fitted adequately by the sum of an exponential decay plus an offset; i.e., a fraction of Rb occlusion is unaffected by temperature in the time scale of the experiment (see the Discussion). An increase in temperature both enhanced the rate at which Rb occlusion was lost (Figure 9A) and decreased the amplitude of the offset. Fitted rate constants for the exponential decay phase at 20, 25, 30, 35, and 40 °C were 0.0075, 0.0115, 0.057, 0.396, and 0.51 min^{-1}, respectively. The Arrhenius plot using these values (Figure 9B) produced a reasonably good line ($R^2 = 0.978$), and the following activation parameters: $\Delta G^{\ddagger} = 19.9$ kcal/mol, $\Delta H^{\ddagger} = 42.7$ kcal/mol, $\Delta S^{\ddagger} = 76.5$ cal mol K^{-1}.

In the experiment in Figure 10, solubilized "19 kDa membranes" were incubated for 2 min at 35 °C in media of pH 5.0−9.0, and the remaining Rb occlusion was then determined. Evidently, Rb occlusion of soluble "19 kDa membranes" is most stable at pH 7.0 (65% of control), with a sharp drop on the acid side of the peak, reaching 1.6% of control at pH 5.0, and a moderate drop on the alkaline side, leveling off at about 30% of control at pH 8.0 and above.

An increase in ionic strength was also damaging. Incubation of the soluble complex with increasing concentrations of choline chloride, up to 500 mM, reduced the Rb occlusion capacity to about 50% of the control (data not shown). The presence of choline chloride prior to solubilization was much more deleterious. As little as 25 mM choline chloride sufficed to reduce the Rb occlusion capacity by 50% (not shown).

DISCUSSION

Protein Components of Soluble Complexes. (a) Rb Occlusion Intact. Solubilization of "19 kDa membranes", in the presence of Rb ions and ouabain, led to the demonstration of the existence of a complex of tryptic fragments containing occluded Rb ions. In principle, it was conceivable that certain fragments of the pump might not be solubilized, and might thus be excluded as components of the cation occlusion domain. In reality, the protein components of the complex were found to contain all fragments of the α, β, and γ subunits present in "19 kDa membranes" (Figures 4−6). The present work verifies directly the inference, based on proteolysis (Shainskaya & Karlish, 1994), of the existence of a Rb-stabilized complex containing all the fragments of α, β, and γ subunits, and strongly supports the notion that different fragments contribute ligating groups to a cation "cage".

Components of the soluble complex (Figure 5B) derived from the α subunit include the fragments containing transmembrane segments M1/M2 (*e*−*g*, 11.7−10.3 kDa), M3/M4 (*i*, 8.6 kDa), M5/M6 (*j*, 8 kDa), and M7/M10 (*c*, 19 kDa), while those derived from the β subunit include the intact subunit (*a*) and its ≈50 kDa (*b*) and 16 kDa (*d*) tryptic fragments [see Capasso et al. (1992)]. It is of interest that

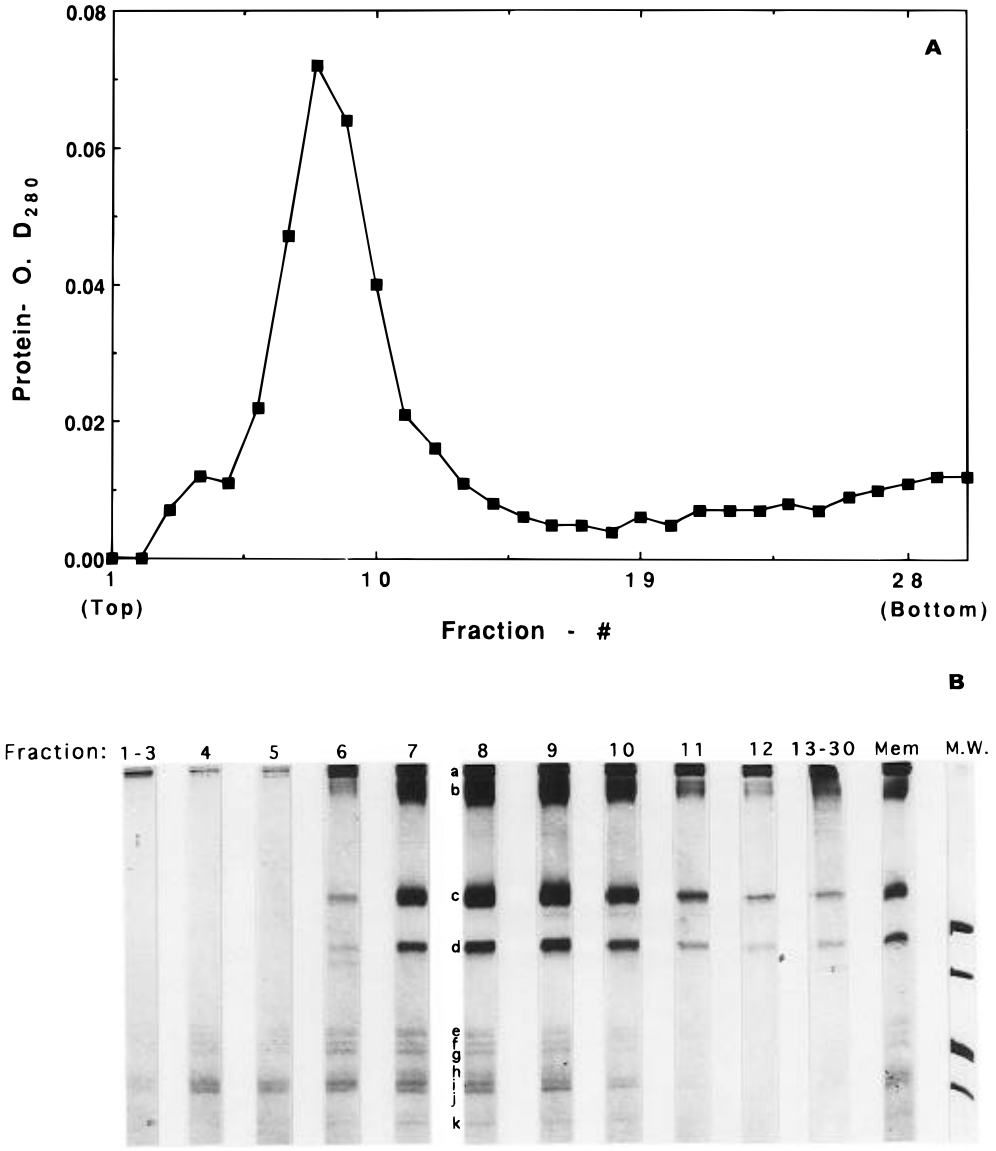

FIGURE 7: Sucrose density gradient profile of "19 kDa membranes" solubilized in the absence of Rb and ouabain. Details are as in Figure 5, except that solubilization and sedimentation were done in the absence of Rb and ouabain. (A) Profile of protein concentration. (B) Fractions resolved on a 16.5% Tricine gel. The figure indicates which fractions were pooled together in each lane. Mem, solubilized "19 kDa membranes"; M.W., molecular mass markers (kDa): 16.9, 14.2, 10.7, 8.2, 6.2; (*a*) β subunit; (*b*) ≈50 kDa fragment; (*c*) 19 kDa fragment; (*d*) 16 kDa fragment; (*e*−*k*) peptides of 11.7−8 kDa.

the γ subunit (*g*, *h*) and a tryptic fragment of γ (*k*) were also associated with the other fragments of the α and β subunits. This finding is consistent with that of Mercer et al. (1993), who showed that the γ subunit is associated with the α and β subunits of Na,K-ATPase. The γ subunit is labeled by photoaffinity derivatives of ouabain, suggesting that it is contiguous with or is part of the binding site of cardiac glycosides (Forbush et al., 1978; Rogers & Lazdunski, 1979). However, no role for the γ subunit in the expression or function of Na,K-ATPase has been identified.

There are two indications that the soluble functional unit of "19 kDa membranes" consists of a monomer containing a single copy of each fragment. (a) Size-exclusion HPLC. The elution time of the major peak in Figure 8 is consistent with a M_r close to that of the denatured α chain or the soluble marker of 128 kDa. Both α (112 kDa) and β (≈60 kDa with sugars) subunits run a little ahead of the positions of soluble proteins of equivalent M_r, due presumably to the extra detergent bound to the hydrophobic regions[see Møller and Le Maire (1993)]. The amount of detergent bound to the soluble complex of fragments could be expected to be similar. Thus, the experiment distinguishes clearly between the presence of monomers and dimers or higher order oligomers of the complex, for any of the latter species should have eluted before the position of the soluble marker of 158 kDa. (b) Specific Rb occlusion. The specific activity of the solubilized "19 kDa membranes" preparation is 20.0 ± 1.2 nmol of Rb/mg of protein. Since two Rb ions are occluded per phosphoenzyme (Shani et al., 1987; Matsui & Homareda, 1982), there should be 10.0 ± 0.6 nmol of soluble active complexes per milligram of protein. This value corresponds to a complex with a mass of 100 ± 6 kDa. The latter value can be compared with the sum of the molecular mass values of the individual components assuming that each fragment is present in a single copy. The estimate is 93 kDa based on the following molecular mass values calculated from the primary sequences [see Table 4 of Shainskaya and Karlish (1994)]: α subunit fragments, Ala68−Arg166, 10.32 kDa; Ile263−Lys352, 9.08 kDa; Gln737−Arg830, 10.32 kDa; Asn831−Tyr1016, 21.82 kDa; β subunit, 34.6 kDa;

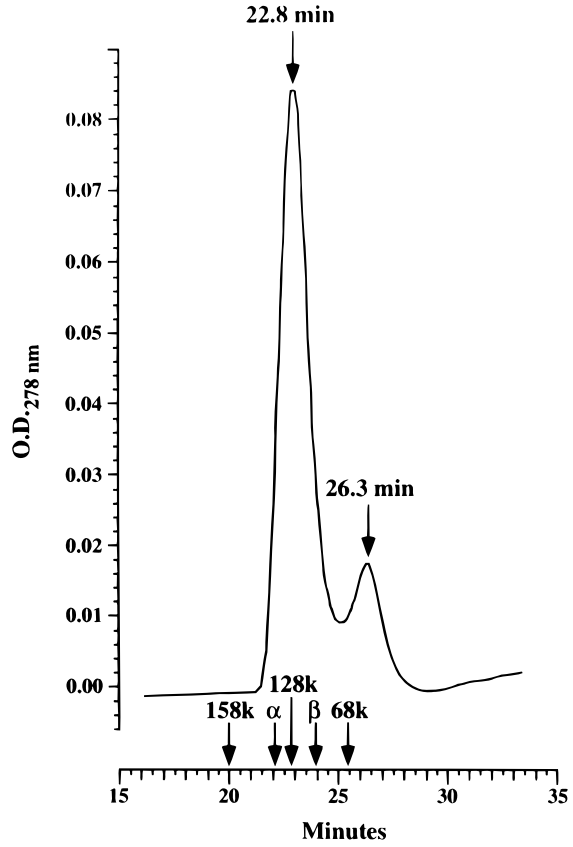

FIGURE 8: Size-exclusion chromatography of solubilized "19 kDa membranes" 40−50 μg of $C_{12}E_{10}$-solubilized "19 kDa membranes", molecular mass markers, or SDS-denatured Na,K-ATPase was injected onto the TSK-3000 SW column, and the protein was eluted at 0.5 mL/min with a running buffer consisting of 30 mM Hepes (Rb), pH 7.0, 10 mM RbCl, 1 mM ouabain, and 0.4 mg/mL $C_{12}E_{10}$. Soluble molecular mass markers (bovine serum albumin monomer and dimer, 68 and 126 kDa, respectively, and aldolase, 158 kDa) were dissolved at 2 mg/mL in the HPLC running buffer without ouabain. Separated α and β subunits were prepared by dissolving renal Na,K-ATPase with 2% SDS, precipitating protein with 4 volumes of methanol, dissolving the precipitate in a solution of 50 mM Tris-HCl, pH 8.0, containing 0.05% SDS, and then adding $C_{12}E_{10}$ to 20 mg/mL.

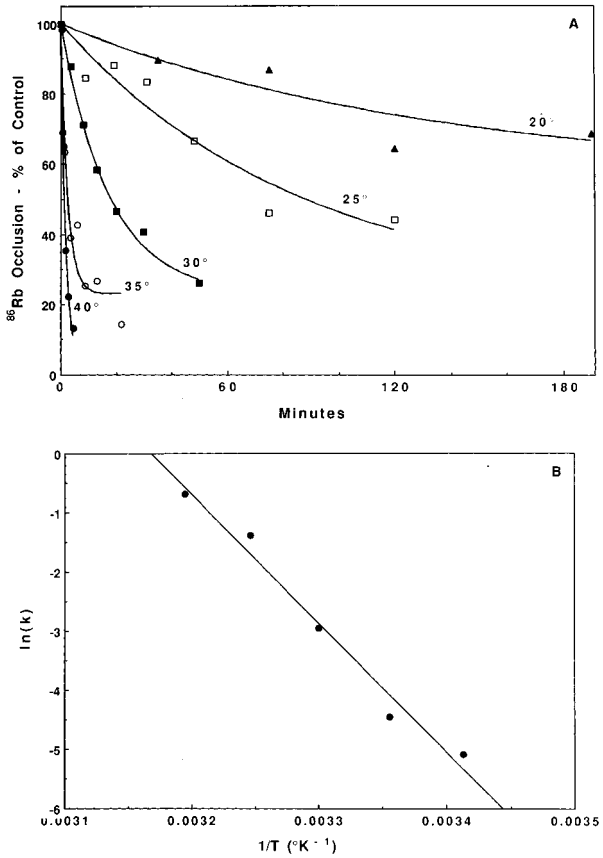

FIGURE 9: Thermal stability of solubilized Rb occlusion activity. (A) Solubilized "19 kDa membranes" were incubated at 20, 25, 30, 35, or 40 °C. 50 μL aliquots were withdrawn at different time intervals, and the occluded Rb was determined. Activities are presented as a percent of the control Rb occlusion activity of a solubilized sample kept on ice. Data were fitted with the equation $Y = (100 - C)\exp(-kt) + C$. Best-fit values for the rate of decay (k) and offset (C) are, respectively: 20 °C, 0.0075 min^{-1}, 55.9%; 25 °C, 0.0115 min^{-1}, 21.4%; 30 °C, 0.057 min^{-1}, 22.6%; 35 °C, 0.396 min^{-1}, 22.9%; 40 °C, 0.51 min^{-1}, 1%. Lines drawn are the fitted curves. (B) Arrhenius plot. For each temperature, the decay rates obtained in the above and a similar experiment were averaged. The fitted value of the slope of the line is −21 763 and the intercept is 68.95 ($R^2 = 0.978$).

and γ subunit, 6.5 kDa [see Mercer et al. (1993)].

(b) Rb Occlusion Inactivated. In the absence of Rb and ouabain, the complex of tryptic fragments dissociated, as revealed by the shift in the position of the small peptides (fragments *e*−*k*) relative to that of the β subunit (*a*), the ≈50, 19, and 16 kDa fragments (fragments *b*−*d*; compare Figures 5B and 7B). Thus, two distinct groups of peptides could be distinguished in this condition.

(A) The β subunit or its ≈50 and 16 kDa fragments remained strongly associated with the 19 kDa fragment even in the absence of Rb and ouabain (Figure 7B). This finding is consistent with evidence that the strongest subunit interactions occur between the extracellular domains of the β and α subunits of both Na,K-ATPase and H,K-ATPase. Interacting residues of the α subunit are located within a stretch of 26 amino acids (Asn894−Ala919) in the loop between M7 and M8 ([Lemas et al., 1994; see also Shin and Sachs (1994)]. Interacting residues of the β subunit appear to be located within the 16 kDa fragment between the cysteines forming the first S−S bridge (Fambrough et al., 1994; Ueno et al., 1995). The importance of extracellular subunit interactions for stability can be appreciated from the fact that their disruption by heat inactivates the enzyme and exposes extracellular loops of the α subunit and also the 16 kDa fragment of the β subunit to proteases (Goldshleger et al., 1995). Heating at 55 °C leads to a change in the native topology of the α subunit in which the M8 and M9 segments are ejected from the membrane (Goldshleger et al., 1995; Arhystarkhova et al, 1995). Rb ions protect against these perturbations.

(B) Two small peptides which include transmembrane segments M3/M4 (*i*) and M5/M6 (*j*), respectively, are the first to dissociate from the solubilized complex in the absence of Rb and ouabain (Figure 7B, fractions 4 and 5) and sediment together on the sucrose gradient. The M1/M2 fragment (*e*, *f*, *g*) and γ subunit (*g*, *h*) occupy a middle position on the gradient. Although we cannot exclude the possibility that all these fragments dissociate randomly from the complex, the finding could indicate the existence of an interaction between the M5/M6 and M3/M4 pairs and a weaker one between them and the M1/M2 fragment and the γ subunit. Initially, the M5/M6 region was suggested to contain a single trans-membrane segment, but several techniques indicate that the sequence (Asn776−Tyr817)

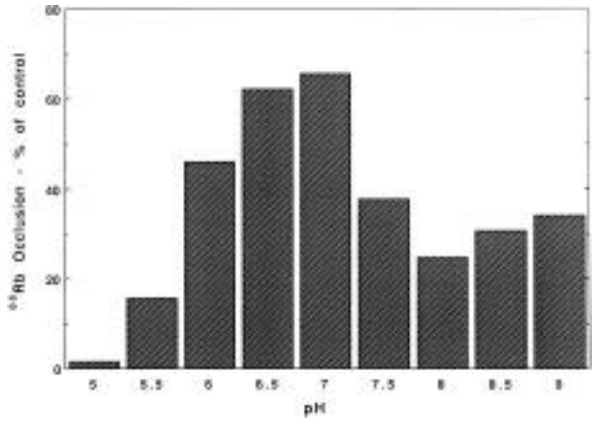

FIGURE 10: Effect of pH on stability of solubilized Rb occlusion activity. Solubilized "19 kDa membranes" (55 μL) were mixed with 9 μL of 20.6 mM MES, 57 mM Bis-Tris-propane/HCl, and 5 mM RbCl + ^{86}Rb (final pH 5.0−9.0 at 35 °C) and were incubated for 2 min at 35 °C. Rb occlusion was determined on two 50 μL aliquots for each pH. Activities are presented as a percent of the Rb occlusion activity of a solubilized sample of "19 kDa membranes" kept on ice.

contains a pair of segments (Karlish et al., 1993; Canfield & Levenson, 1993; Ning et al., 1993b; Yoon & Guidotti, 1994). However, the M5/M6 pair is atypical in that the segments are shorter than the standard 20−22 residues, they contain several charged residues which are important for interaction with the cations (Jewell-Motz & Lingrel, 1993; Vilsen, 1995), and M5 contains several proline residues which would distort an α helix. In an *in vitro* translation assay, sequences representing M5, M6, and M7 of H,K-ATPase act neither as signal anchor nor as stop transfer signals, suggesting that they may be inserted into the membrane post-translationally (Bamberg & Sachs, 1994). Shin and Sachs (1994) also reported that a carbonate wash at pH 10.0 selectively removes the M5/M6 segment from extensively trypsinized membranes. The corresponding M5/M6 fragment of Na,K-ATPase was shown recently to be released from "19 kDa membranes" in the absence of Rb ions, but it remained associated with the membrane in the presence of occluded Rb ions (Lutsenko et al., 1995). The result in Figure 7B is consistent with all these findings, and indicates that, in the absence of occluded Rb ions and ouabain, association of the smaller fragments containing M5/M6, M3/M4, and also M1/M2 and the subunit with the 19 kDa fragment (M7−M10) and the β subunit, is weaker than that between the 19 kDa fragment (M7−M10) and the β subunit themselves.

Factors Affecting the Stability of the Cation Occlusion Domain. The points discussed below provide evidence that the Rb occlusion domain is normally stabilized by interactions both within the membrane domain and between hydrophilic segments of the protein outside the membrane. This concept is consistent with conclusions based on thermal inactivation and proteolysis of "19 kDa membranes" (Or et al., 1993; Shainskaya & Karlish, 1994; Lutsenko & Kaplan, 1994) and intact Na,K-ATPase (Goldshleger et al., 1995). Proteolysis of Ca-ATPase led to similar conclusions (Juul et al., 1995). By contrast, bacterial membrane proteins, such as bacteriorhodopsin, with little of the protein outside the membrane, appear to be stabilized primarily by interactions between side chains of trans-membrane segments (Lemmon & Engelman, 1994).

(a) Detergent versus Membrane Phospholipid. During solubilization, the nonionic detergent is thought to replace the membrane phospholipid, but the detergent is not a perfect mimic of the native membrane lipid. At 0 °C, Rb occlusion activity of "19 kDa membranes" was solubilized by $C_{12}E_{10}$, increasing to an optimum at a detergent to protein weight ratio of 1.8 w/w, but it was then progressively inactivated, falling to 16% of control at a ratio of 8 w/w (Figure 2). A similar phenomenon of solubilization followed by inactivation of native Na,K-ATPase has been described previously (Esmann & Skou, 1984; Brotherus et al., 1983; Esmann, 1985). The detergent to protein weight ratios are similar, when corrected for the ratio of molecular masses of the protein in "19 kDa membranes" and native Na,K-ATPase (about 2:3). Brotherus et al. (1979) studied inactivation of Na,K-ATPase from eel electroplax by different detergents, and found that each detergent displays a characteristic threshold for inactivation, proportional to the lipid concentration in the membrane. At higher temperatures, the threshold is lowered, rendering the solubilized enzyme temperature-sensitive (Esmann & Skou, 1984). The latter finding is consistent with our observation that at 22 °C no more than 46% of the original Rb occlusion activity could be solubilized (Figure 2).

The present work shows that detergent-solubilized "19 kDa membranes" are much less stable than intact "19 kDa membranes". Two strong indications for this conclusion are that, after solubilization, Rb occlusion is no longer maintained in the absence of Rb and ouabain, and even in the presence of Rb and ouabain, Rb occlusion of solubilized "19 kDa membranes" is inactivated at temperatures (e.g., 40 °C, see Figure 9) at which intact "19 kDa membranes" are quite stable (Or et al., 1993; Shainskaya & Karlish, 1994; Nesatyi, Shainskaya, and Karlish, unpublished data). The implication of all these findings is that protein−phospholipid or protein−protein interactions within the membrane domain are normally important stabilizing factors.

(b) Rb Ions and Ouabain. The stabilization of Rb occlusion by the presence of both Rb ions and ouabain during solubilization of "19 kDa membranes" can be understood as a special case of a general mechanism whereby occluded Rb ions protect against structural perturbations. For example, Rb ions strongly protect "19 kDa membranes" against thermal inactivation of Rb occlusion (Or et al., 1993) and further proteolytic digestion (Karlish et al., 1990; Capasso et al., 1992; Shainskaya & Karlish, 1994). Recently, we have proposed (Shainskaya & Karlish, 1996) that inactivation of Rb occlusion following chymotrytpic truncation of the β subunit, or inactivation induced by DTT [see Lutsenko and Kaplan (1993)], is caused by reduction in Rb affinity, dissociation of occluded Rb ions, and then thermal inactivation. This sequence of events constitutes a general mechanism for inactivation of cation occlusion. In the present solubilization experiments, partial preservation of occlusion was achieved by raising the Rb concentration to 5 mM even without ouabain (Table 1). Rb occlusion on intact "19 kDa membranes" is saturated at low concentrations ($K_{0.5} \approx 50$ μM; Karlish et al., 1990). Thus, a reasonable hypothesis could be that solubilization was associated with a large decrease in the apparent Rb binding affinity due to destabilization of the complex by the detergent, and high concentrations of

Rb ions would be required to protect against such destabilization. Ouabain greatly reduced the rate of dissociation of Rb ions from "19 kDa membranes" (Figure 1), and at nonsaturating Rb concentrations, ouabain greatly stabilizes Rb occlusion against thermal inactivation (Nesatyi, Shainskaya, and Karlish, unpublished data). A major role of ouabain is probably to counteract the increased rate of Rb dissociation induced by the detergent, and maintain the stabilizing protein−protein interactions by keeping the Rb ions occluded. Ouabain might also stabilize the complex directly by binding to several fragments and reducing their tendency to become disorganized.

(c) Temperature. A detailed study of the kinetics of thermal inactivation of Rb occlusion in intact "19 kDa membranes" shows that it can be described by a three-state model, A↔I→D, in which a reversible equilibrium between an active (A) and an inactive (I) state precedes an irreversible step to a denatured (D) state [Nesatyi, Shainskaya, and Karlish, unpublished data; see Lumry and Eyring (1954)]. The kinetics of irreversible thermal inactivation of the solubilized Rb occlusion activity could not be analyzed in such detail because the measurement is less accurate. Inactivation curves were fitted to the sum of an exponential process plus an offset (Figure 9A), although it is probable that, in reality, the process should be described as the sum of two exponentials. The fitted rate constants for the exponential phase represent composites of the rate constants of the different steps of the process. The Arrhenius plot (Figure 9B) is linear and provides a composite measure of the activation energy parameters for the whole process of irreversible inactivation. The estimated activation enthalpy and entropy ($\Delta H^{\ddagger}$, 42.7 kcal/mol; $\Delta S^{\ddagger}$, 76.5 cal mol^{-1} K^{-1}) are consistent with the notion that a large number of noncovalent interactions are disrupted in the inactivation process and the complex of fragments becomes disorganized. The $\Delta H^{\ddagger}$ (42.7 kcal/mol) is comparable to that for thermal denaturation of lysozyme, a small globular protein ($\Delta H^{\ddagger}$, 47.8 kcal/mol; Segawa & Sugihara, 1984). The latter authors suggested that the three-dimensional structure is stabilized by cooperative interactions distributed throughout the protein and therefore denaturation requires multiple perturbations.

It is of interest to compare the estimated activation parameters for solubilized "19 kDa membranes" ($\Delta G^{\ddagger}$, 19.9 kcal/mol; $\Delta H^{\ddagger}$, 42.7 kcal/mol; $\Delta S^{\ddagger}$, 76.5 cal mol^{-1} K^{-1}) with those reported by Jørgensen and Andersen (1986) for thermal inactivation of native Na,K-ATPase solubilized with $C_{12}E_8$ in the presence of KCl ($\Delta G^{\ddagger}$, 25.3 kcal/mol; $\Delta H^{\ddagger}$, 87.2 kcal/mol; $\Delta S^{\ddagger}$, 200 cal mol^{-1} K^{-1}). The difference in $\Delta G^{\ddagger}$ is a measure of the fact that the soluble "19 kDa membranes" are much less stable than soluble Na,K-ATPase. The $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for "19 kDa membranes" are much less than for native enzyme, presumably because "19 kDa membranes" are stabilized by fewer noncovalent interactions and are less ordered. The overall conclusion is that the cytoplasmic domain of the native enzyme, which is absent in "19 kDa membranes", is important for stabilizing the occlusion domain within the membrane.

(d) pH and Ionic Strength. The effects of pH (Figure 10) and ionic strength on the thermal lability of solubilized "19 kDa membranes" indicate that stabilizing interactions between the hydrophilic domains must be important. Such interactions could include H-bonds or salt bridges between negatively charged aspartates, glutamates, and arginines or protonated lysines and histidines. Many charged residues are located close to trans-membrane segments near presumed points of entry into or exit from the lipid. In this way, interactions outside the membrane could affect the stability of the cation occlusion sites located within the membrane.

Potential Use of Soluble "19 kDa Membranes" for Cross-Linking Experiments. In principle, one might determine the proximity of neighboring fragments in "19 kDa membranes" by covalent cross-linking. However, intramolecular cross-linking using intact "19 kDa membranes" is likely to be complicated by the very high surface concentration of the protein (1 g of Na,K-ATPase per milliliter of lipid; Jørgensen, 1982). This factor should favor competing intermolecular cross-linking between adjacent complexes rather than intramolecular cross-linking. Solubilization of "19 kDa membranes" prior to cross-linking might overcome this obstacle due to the possibility of diluting the protein at will. Preservation of Rb occlusion and ouabain binding, in the soluble complex, and also the ordered dissociation of the two occluded Rb ions provide important criteria that the trans-membrane segments retain the original arrangement. The soluble preparation is now being used for cross-linking studies (Or, Goldshleger, and Karlish, unpublished data).

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