Interactions between Fragments of Trypsinized Na,K-ATPase Detected by Thermal Inactivation of Rb\(^+\) Occlusion and Dissociation of the M5/M6 Fragment*

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Alla Shainskaya‡, Victor Nesaty, and Steven J. D. Karlish§

From the Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

This work provides evidence for interactions between fragments of “19-kDa membranes,” a trypsinized preparation of Na,K-ATPase that retains cation occlusion and ouabain binding. Previously, we reported rapid thermal inactivation of Rb\(^+\) occlusion at 37 °C (Or, E., David, P., Shainskaya, A., Tal, D. M., and Karlish, S. J. D. (1993) J. Biol. Chem. 268, 16929–16937). We describe here the detailed kinetics of thermal inactivation. In the range 25–35 °C, a two-step model (N \(\rightarrow\) U \(\rightarrow\) I, where N is the native species, U is the reversibly unfolded intermediate, and I is the irreversibly denatured form) fits the data. Reversibility of inactivation has been observed at 25 °C, consistent with the model. At 37 °C and higher temperatures, the data can be fitted to a simple mechanism N \(\rightarrow\) U, i.e. U is not significant as an intermediate. Occluded cations (Na\(^+\), Rb\(^+\), K\(^+\), Tl\(^+\), NH\(_4\)\(^+\), and Ca\(^2+\)) and ouabain protect strongly against thermal inactivation. Ca\(^2+\), Ba\(^2+\), and La\(^3+\) ions do not protect. Proteolysis experiments provide independent evidence that disorganization can occur in stages, first in transmembrane segments and then in extra-membrane segments of the fragments. Analysis of selective dissociation of the M5/M6 fragment at 37 °C (Lutsenko, S., and Kaplan, J. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7936–7940), using a specific antibody, showed that inactivation of Rb\(^+\) occlusion precedes dissociation of the fragment, and only ~50% of the fragment is released when occlusion is fully inactivated. In the presence of Ca\(^2+\) ions, occlusion is inactivated, but the M5/M6 fragment is not released. The experiments demonstrate that occlusion is inactivated by disruption of interactions between fragments of 19-kDa membranes, and only then does the M5/M6 fragment dissociate. Interactions between the M5/M6 and M7/M10 fragments seem to be essential for maintenance of Rb\(^+\) occlusion.

Recent studies of structure-function relations of the higher P-type active cation pumps have focused on the characterization of functional sites, for ATP and cations or inhibitors such as the cardiac glycoside, or on experimental determination of transmembrane topology (3). The consensus number of transmembrane segments in \(\alpha\)-subunits of Na,K-, H,K-, and Ca-ATPases is 10, and therefore, the focus of attention is now shifting to the question of their arrangement in the plane of the membrane. Site-directed mutagenesis or chimeric molecules are being used intensively to characterize residues involved in cation occlusion. Site-directed mutations suggest that carboxyl and other oxygen-containing side chains of residues within transmembrane segments M4–M6, and perhaps M3, ligate the occluded cations (4–6). Thus, the transmembrane \(\alpha\)-helices are arranged so as to create the cation occlusion “cage.”

A complementary biochemical approach for the study of cation sites and the organization of transmembrane segments utilizes a preparation of renal Na,K-ATPase extensively digested with trypsin (7, 8). This preparation, referred to as “19-kDa membranes,” consists of well defined fragments of the \(\alpha\)-subunit containing transmembrane segments M7–M10 (apparent molecular mass of ~19 kDa); the pairs M1/M2, M3/M4, and M5/M6 (apparent molecular mass of 8–11.7 kDa); and the \(\beta\)-subunit partially split into a 16-kDa N-terminal and an ~50-kDa C-terminal fragment. Cation occlusion and ouabain binding are intact, but ATP binding is absent. These features indicate that cation occlusion sites are located within transmembrane segments. Occluded cations (K\(^+\), Na\(^+\), and congeners) (7, 8) and also ouabain (9) strongly protect the 19-kDa and smaller fragments of the \(\alpha\)-subunit and also the 16-kDa fragment of the \(\beta\)-subunit against trypsin. In the absence of occluded cations or if Rb\(^+\) ions are displaced by Ca\(^2+\) ions, Rb\(^+\) occlusion is destroyed, and all the fragments are digested further to limit membrane-embedded peptides (10). The segments digested away from 19-kDa membranes consist of tails and loops between transmembrane segments outside the membrane. These findings indicated that the fragments interact as a complex in which several transmembrane segments cooperate to occlude the cations, consistent with conclusions based on mutations. Occluded Rb\(^+\) or Na\(^+\) or ouabain induces interactions between fragments, stabilizing a compact structure that is inaccessible to trypsin. Recently, we have demonstrated directly the existence of a detergent-solubilized complex of all the fragments containing occluded Rb\(^+\) ions and bound ouabain (11).

One objective of this work has been to investigate an observation that incubation of 19-kDa membranes at 37 °C in the absence of occluded cations leads to rapid loss of the ability to occlude Rb\(^+\) ions (1). Occluded Rb\(^+\) or Na\(^+\) ions protect against this thermal inactivation as well as against further trypsic digestion of the fragments. Indeed, trypsin digests only thermally inactivated 19-kDa membranes, suggesting that the process involves disorganization of the interacting fragments (10). A similar implication has come from an observation that ouabain binding and electrogenic Na\(^+\) binding are thermally inactivated at the same rate (12). In recent experiments utilizing chymotrypsin to demonstrate a specific role of the cytoplasmic tail of the \(\beta\)-subunit, loss of Rb\(^+\) occlusion has also been found to be a result of thermal inactivation (13). We have also...
proposed that loss of Rb+ occlusion, following reduction of S–S bridges in the β-subunit (14), represents accelerated thermal inactivation of a destabilized structure. Thermal inactivation of Rb+ occlusion appears to be a general phenomenon accompanying structural perturbations of 19-kDa membranes and can be used as a tool to investigate the structure of the cation-binding domain, the strength of the interactions between different fragments, and the effect of ligands or substrates on stability. Thermal inactivation of the native Na,K-ATPase, with protection by K+ and Na+ ions, was described earlier (15, 16), but 19-kDa membranes, which lack the cytoplasmic loops of the α-subunit, are much more thermostable than native enzyme and represent a simpler system to analyze the factors determining the stability and interactions between transmembrane segments.

Lutsenko and Kaplan (2) have shown recently that, upon incubation of 19-kDa membranes at 37 °C in the absence of Rb+ ions or ouabain, the fragment containing the M5/M6 segment is selectively and irreversibly released into the medium. Dissociation of the M5/M6 fragment is associated with partial inhibition of Rb+ occlusion, and loss of Rb+ occlusion has been described as accompanying (2) or being caused by (17) dissociation of the M5/M6 fragment. Since occluded Rb+ ions prevent dissociation of the M5/M6 fragment and also protect against thermal inactivation, it has become necessary to characterize the relationship between the two phenomena.

**EXPERIMENTAL PROCEDURES**

Na,K-ATPase was prepared from fresh pig kidney red outer medulla by the rapid procedure described by Jørgensen (18). Protein was determined by the method of Lowry et al. (19), and ATPase activity was determined as described previously (18). Specific activity was in the range 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). Standard conditions for preparation of tryptic 19-kDa membranes were described by Capasso et al. (8). After digestion, membranes were washed, suspended, and stored in standard medium (25 mm imidazole, pH 7.5, and 1 mM EDTA), to which 2 mM RbCl was added.

**Rb+ Occlusion Assay—**The Rb+ occlusion assays were performed as described by Shani et al. (20). The medium contained, in a volume of 20–50 μl, 1 mM RbCl plus ~5 × 10^4 cpm 86Rb+; 12.5 mm imidazole, pH 7.5, 0.5 mM EDTA, and 10–20 μg of 19-kDa membranes.

**Thermal Inactivation of 19-kDa Membranes—**Membranes were centrifuged and suspended in standard medium containing 0.1 mM RbCl and 1% Triton X-100 for 1 h. They were then washed again and suspended in an Rb+ “free” medium. The final free Rb+ concentration was estimated to be <1 μM. 19-kDa membranes were incubated at 0.5–2 mg/ml, under the conditions indicated in the figure legends, in a thermostatically controlled water bath. At the indicated times, aliquots were placed on ice; reaction medium containing 1 mM RbCl plus ~5 × 10^4 cpm 86Rb+ was added; and Rb+ occlusion was measured after a 60-min incubation at 0 °C or 5 min at 20 °C. Experimental points represent averages of duplicate samples. Variability between duplicates was <10%.

**Dissociation of the M5/M6 Fragment—**19-kDa membranes (150 μg/sample) were diluted with 1 ml of standard medium and centrifuged at 250,000 × g for 1 h, and the pellet was resuspended in ice-cold medium containing 10 mM Tris, 10 mM RbCl, or 1 mM CaCl2. Phenylmethylsulfonyl fluoride, thioglycolate, and molecular mass markers (2.5–16.9 kDa) were from Sigma. Choline chloride (recrystallized from hot ethanol) was from Supelco. Thallous acetate, lithium chloride, and magnesium chloride were from Fluka. TPCK-treated trypsin (bovine pancreas, 2.4 units/mg) was from Worthington. For SDS-polyacrylamide gel electrophoresis, all reagents were of electrophoresis grade and were from Bio-Rad. Polyvinylidene difluoride paper was from Millipore Corp. Thallous acetate, lithium chloride, and magnesium chloride were from BDH (Poole, United Kingdom). Cesium chloride was from Fisher. Ammonium and potassium chlorides were from Merck. Lanthanum chloride heptahydrate was from Aldrich.

**RESULTS**

**Kinetics of Thermal Inactivation of Rb+ Occlusion—**Fig. 1 shows representative time courses of thermal inactivation of Rb+ occlusion in the temperature range 25–45 °C and in the presence of 0.1 mM RbCl. The samples were centrifuged at 250,000 × g for 1 h. Pellets were resuspended in standard medium containing 2 mM RbCl. Prior to SDS-polyacrylamide gel electrophoresis, pellets were resuspended in standard medium and dissolved in 4% SDS, and protein was precipitated by addition of 4 volumes of ice-cold methanol and stored overnight at −20 °C. The delipidated protein was collected by centrifugation for 30 min at 10,000 rpm in a Sorvall centrifuge, dried under a stream of nitrogen, and dissolved in 10% SDS or the sample buffer. The supernatants were collected, lyophilized, and dissolved in the sample buffer. Equal amounts of delipidated membrane protein (~100 μg for staining and 10 μg for immunoblotting) and equivalent amounts of supernatant and pellet were applied per lane of 10% gels.

**Gel Electrophoresis—**Triticine/SDS-polyacrylamide gel electrophoresis was done essentially according to Schägger and von Jagow (21) using either 1-mm-thick 10% gels (10% T and 3% C separating gel (11.5 cm) plus 4% T stacking gel (1.5 cm)) or 1-mm-thick 16.5% gels (16.5% T and 6% C separating gel (20 cm), 10% T spacing gel (2 cm), and 4% T stacking gel (1.5 cm)). Full details of the electrophoresis procedure are given by Capasso et al. (8). Staining of transparencies of photographs of gels was performed with a Molecular Dynamics 300A computing densitometer.

**Immunoblots—**Immunoglobulins raised against the synthetic peptide Leu^{135–181}Gln^{188} were supplied by Dr. J. V. Møller (Aarhus University, Aarhus, Denmark), and anti-Lys^{1012–1016}Ty^{1016} was referred to as anti-KETTY, was supplied by Dr. J. Kyte (University of California at San Diego, La Jolla, CA). Rabbit antiserum, prepared as described (22), were raised against fragments of 19-kDa membranes (7, 8) and included (i) anti-M1/M2, prepared from a 11.7-kDa fragment (Asp^{86}–Arg^{176}) containing M1 and M2; (ii) anti-β, prepared from a 18-kDa fragment (Ala^{37}–Arg^{142}) of the β-subunit; and (iii) anti-γ. Anti-peptide antibodies were also raised against the synthetic peptides Leu^{137}–Asn^{168} and Leu^{362}–Pro^{376}, coupled to keyhole limpet hemocyanin. Antibodies were diluted 1:100–400 in a solution of 1.5% (w/v) bovine serum albumin in Tris-buffered saline solution. Samples were delipidated, separated on 16% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblot analysis was described previously in detail by Capasso et al. (8).

**Calculations—**Best fit parameters of theoretical equations to experimental data were calculated by nonlinear regression analysis using the Mathematica and MatLab software programs. First-order rate constants for the thermal inactivation process were determined from least-squares fits of the data to Equation 4 in the “Appendix.” The error estimates of the parameters are calculated by linear approximation (24).

**Calculation of Activation Parameters—**Activation parameters were calculated as described by Huang (25). Activation energy (Ea) values were estimated from slopes of Arrhenius plots (ln k versus 1/T). Activation enthalpies (ΔH‡) for each temperature were calculated from the relation ΔH = E−RT, where R (8.314 J·mol−1·K−1) is the universal gas constant and T is the absolute temperature. Activation free energies (ΔG‡) were calculated according to Equation 1.

$$\Delta G^\circ = -RT \ln \frac{h}{k_B T} \quad (\text{Eq. 1})$$

where k is the rate constant, h (6.6265 × 10−27 J s) is the Planck constant, and K is (1.3805 × 10−23 J·K−1) is the Boltzmann constant. Activation entropies (ΔS‡) were then calculated according to Equation 2.

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad (\text{Eq. 2})$$

Materials—96RbCl was obtained from NEN Life Science Products. Dowex 350-X8 (100 mesh, H-form; converted to the Tris form before use) was obtained either from Sigma or Fluka. Trypsin inhibitor (type 1-S from soya bean), bovine serum albumin (fraction V), phenylmethylsulfonyl fluoride, thioglycolate, and molecular mass markers (2.5–16.9 kDa) were from Sigma. Choline chloride (recrystallized from hot ethano- l) was obtained from Fluka. TPCK-treated trypsin (bovine pancreas, 2.4 units/mg) was from Worthington. For SDS-polyacrylamide gel electrophoresis, all reagents were of electrophoresis grade and were from Bio-Rad. Polyvinylidene difluoride paper was from Millipore Corp. Thallous acetate, lithium chloride, and magnesium chloride were from BDH (Poole, United Kingdom). Cesium chloride was from Fisher. Ammonium and potassium chlorides were from Merck. Lanthanum chloride heptahydrate was from Aldrich.

1 The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis(hy- droxymethyl)ethyl]glycine; TPCK, tosylphenylalanyl chloromethyl ketone.
absence of Rb$^+$ ions. Because 19-kDa membranes are most stable when stored in medium containing Rb$^+$ ions, removal of contaminating Rb$^+$ ions by thorough washing of the membranes (see "Experimental Procedures") was essential to ensure maximal reproducibility of time courses for different preparations. If, for example, the membranes were centrifuged only once and then resuspended in nominally Rb$^+$ free solutions, the occlusion was not fully inactivated at 37 °C, and the final level varied in different experiments. Reports of incomplete thermal inhibition (2, 26) of proteolyzed dog kidney enzyme might be explained by insufficient washing of the membranes.

The time courses in Fig. 1 appeared to be biphasic, at least in the range 25–35 °C, and the kinetics were therefore analyzed using the two-step model of enzyme denaturation proposed by Lumry and Eyring (27) (Equation 3),

$$
\begin{align*}
N & \xrightarrow{k_1} U \\
U & \xrightarrow{k_2} I
\end{align*}
$$

(Eq 3)

where N is the active native enzyme, U is the reversibly unfolded inactive enzyme, and I is the irreversibly inactivated enzyme. The model is described by the three rate constants $k_1$, $k_2$, and $k_3$. Equation 4 in the “Appendix” assumes that the remaining occlusion activity reflects the value of N and describes a double exponential inactivation kinetic. The continuous lines in Fig. 1 represent best fits to Equation 4. Best fit values of $k_1$, $k_2$, and $k_3$ and also the amplitudes of the two phases ($A_1$ and $A_2$) were calculated and collected together in Table I. In the range 25–35 °C, the double exponential kinetic provided a good fit to the data. At 40 and 45 °C, the kinetics could not be distinguished from a single exponential process due to the shift of the relative amplitudes of the two phases. Most curves at 37 °C were also fitted adequately by a single exponential decay (data not shown).

Although values of $k_1$, $k_2$, and $k_3$ could be obtained only over a limited range of temperatures and the fitting errors are significant, we have estimated activation parameters ($\Delta H^\ddagger$ and $\Delta S^\ddagger$) from the Arrhenius plots, for the values are informative with respect to the mechanism of thermal inactivation (Fig. 2 and Table II). The positive values of $\Delta H^\ddagger$ and $\Delta S^\ddagger$ for $k_1$ and $k_3$ reflect the fact that $k_1$ and $k_3$ rose as the temperature was raised. However, $k_2$ did not change appreciably between 25 and 35 °C (Table I), and thus, $\Delta H^\ddagger$ is near to zero, whereas $\Delta S^\ddagger$ has a negative value. The data are consistent with the notion that $k_1$ and $k_3$ represent disorganization processes but that $k_2$ represents a reorganization process (see “Discussion”).

The Lumry-Eyring mechanism assumes the existence of a reversible equilibrium between the N and U states, followed by
Previously, we reported that Rb$^+$ (see “Discussion”). The Rb$^+$ occlusion of the samples was determined in the presence of 5 mM RbCl plus $^{86}$Rb$^+$ at times from 0 to 24 h (see “Experimental Procedures”).

Protection against Thermal Inactivation by Occluded Cations—Previously, we reported that Rb$^+$ or Na$^+$ ions protect against thermal inactivation, but aromatic guanidinium and isothiouronium derivatives, which act as competitive Na$^+$ antagonists, do not protect (1, 28). In Fig. 4, a number of cations were tested for protective effects at 37 °C. Rb$^+$, K$^+$, Tl$^+$, Cs$^+$, NH$_4^+$, and Na$^+$ ions protected fully against thermal inactivation under these conditions. The $K_m$ for Rb$^+$ ions for protection was 89 μM (data not shown). Li$^+$ ions were ineffective under these conditions, although in other experiments, Li$^+$ ions at 50 mM were able to protect against thermal inactivation at 30 °C.² La$^{3+}$ ions, which compete with Rb$^+$ or Na$^+$ ions (29), and Ba$^{2+}$ and Ca$^{2+}$ ions, which also compete with Rb$^+$ ions in 19-kDa membranes (10), are ineffective. La$^{3+}$ and Ca$^{2+}$ ions, like the aromatic guanidinium and isothiouronium derivatives (1, 28), do not appear to be occluded (10, 29). Thus, it appears that only cations that can be occluded (K$^+$, Na$^+$, and congeners) can protect against thermal inactivation.

The ability of occluded Rb$^+$ ions to stabilize against thermal inactivation was analyzed in more detail in Fig. 5, which presents representative time courses of loss of occlusion in the presence of a very high concentration of Rb$^+$ ions (50 mM). 50 mM RbCl was used because, in control experiments, the high concentration was found to be necessary to saturate occlusion at 55 °C (data not shown). A striking feature is that occlusion was significantly inactivated only at 45 °C and higher temperatures. Thus, Rb$^+$ ions greatly slowed down thermal inactivation, the rate at 45 °C being about the same as at 25 °C in the absence of Rb$^+$ ions (Fig. 1). The inactivation curves demonstrated biphasic kinetics in the range 45–50 °C, and again the lines represent best fits to Equation 4 in the “Appendix.” At

\[\begin{align*}
\text{FIG. 3. Reactivation of Rb}^+ \text{ occlusion after inactivation at 25 °C.} \\
\text{19-kDa membranes were suspended in standard medium and incubated at 25 °C for 30 min or at 37 °C for 1.5 min. At the indicated times, aliquots were withdrawn and placed on ice. The Rb}^+ \text{ occlusion of the samples was determined in the presence of 5 mM RbCl plus } ^{86}\text{Rb}^+ \text{ at times from 0 to 24 h (see “Experimental Procedures”).}
\end{align*}\]

\[\begin{align*}
\text{FIG. 4. Selectivity of cations in protecting 19-kDa membranes against thermal inactivation.} \quad \text{70 μg of 19-kDa membranes were incubated at 37 °C for 5 min with 2 mM RbCl (Rb), 10 mM KCl (K), 30 mM TICl}_2\text{COO}^-(Ti), 50 mM CsCl (Cs), 50 mM NH}_4\text{Cl, 150 mM NaCl (Na), 50 mM LiCl (Li), 100 mM LaCl}_3\text{(La), 2 mM BaCl}_2\text{(Ba), or 5 mM CaCl}_2\text{(Ca). The control contained no addition and was kept on ice. After incubation, 10 mM RbCl was added, and the membranes were centrifuged and resuspended in standard medium. } ^{86}\text{Rb}^+ \text{ was then added, and occlusion was measured. Protein concentrations of resuspended samples were standardized by comparing } A_{280}\text{.}
\end{align*}\]

\[\begin{align*}
\text{FIG. 5. Time course of inactivation of Rb}^+ \text{ occlusion in presence of Rb}^+ \text{ ions. 19-kDa membranes were incubated at the indicated temperatures in standard medium containing 50 mM RbCl plus } 10 \times 10^6 \text{ cpm } ^{86}\text{Rb}^+. \text{ At the indicated times, samples were withdrawn and transferred to Dowex columns.}
\end{align*}\]

\[\begin{align*}
^2 \text{E. Or, unpublished data.}
\end{align*}\]
52 °C. Fitted rate constants are presented in Table III. Because values for $k_1$, $k_2$, and $k_3$ were obtained only over a 5 °C temperature span (45–50 °C) and the fitting errors are large, it was not possible to obtain reliable quantitative values of activation parameters ($\Delta H^\ddagger$ and $\Delta S^\ddagger$) for comparison with those in the absence of Rb$^+$ ions. Nevertheless, comparison of the fitted rate constants, with or without Rb$^+$ ions, revealed important qualitative features of the mechanism of action of the Rb$^+$ ions (see “Discussion”).

**Protection against Thermal Inactivation by Ouabain—**Ouabain binds tightly to 19-kDa membranes in the presence of Mg$^{2+}$ ions ($K_{b,3} \approx 10 \text{mM} \text{MgCl}_2$) (12) and protects the 19-kDa fragment against tryptic digestion (9). Fig. 6 demonstrates that ouabain also protects against thermal inactivation. Ouabain plus Mg$^{2+}$ ions inhibited Rb$^+$ occlusion to ~37% of the control level, thus complicating the experiment. Nevertheless, in the sample incubated at 37 °C for 5 min with ouabain plus Mg$^{2+}$ ions, the Rb$^+$ occlusion was ~88% of that in the unincubated sample. This value can be compared with the Rb$^+$ occlusion level of only 20% of control for the sample incubated at 37 °C without ouabain plus Mg$^{2+}$. Other control experiments showed that ouabain alone ($K_{b,3} \approx 1 \text{mM}$) slowed down the rate of Rb$^+$ occlusion, but did not inhibit the equilibrium level of Rb$^+$ occlusion after incubation of 19-kDa membranes with reaction medium for 1 h at 20 °C (data not shown). Therefore, protection experiments could also be done conveniently in the absence of Mg$^{2+}$ ions. Fig. 6 shows that ouabain (5 mM) without added Mg$^{2+}$ ions was indeed able to protect strongly against thermal inactivation.

Fig. 7 (A and B) presents a comparison of protection at 37 °C by Rb$^+$ ions (0.5 mM) and ouabain (5 mM), with both ligands being present at close to saturating concentrations. The time courses of thermal inactivation were similar in the presence of either 0.5 mM Rb$^+$ ions or 5 mM ouabain and were much slower than in the absence of either ligand. The combination of 5 mM ouabain with 0.5 mM Rb$^+$ was even more effective. In Fig. 7B, we tested whether the additional protection by ouabain could be observed even in the presence of a very high concentration of Rb$^+$ ions. As seen in Fig. 7B, this was found to be the case at the elevated temperature of 47 °C.

**Sensitivity of Fragments to Proteolysis Associated with Thermal Inactivation—**Previously, we reported that, following irreversible thermal inactivation of Rb$^+$ occlusion at 37 °C, all fragments of 19-kDa membranes can be digested further by trypsin or chymotrypsin to limit membrane-embedded peptides (10, 13). The experiment in Fig. 8 examined the accessibility of the fragments to trypsin during a 30-min digestion at 25 °C, the condition for irreversible inactivation (Fig. 3). Rb$^+$ occlusion was inactivated to 60% of the control level in the sample without Rb$^+$ and trypsin. The result was that the fragments were insensitive to proteolysis and remained essentially intact. Trypsin clipped three fragments known to contain the M1/M2 fragment (8) both in the absence and presence of Rb$^+$ ions (arrows). These clips are unrelated to the reversible thermal inactivation since occlusion was not inactivated in the presence of Rb$^+$ ions at 25 °C. No cleavage of other fragments was detected in the absence or presence of Rb$^+$ ions. Thus, the extra-membrane tails or loops were essentially inaccessible to trypsin under conditions of reversible thermal inactivation.

**Dissociation of the M5/M6 Fragment and Thermal Inactivation of Rb$^+$ Occlusion—**Figs. 9 and 10 present immunoblots of 19-kDa membranes and supernatants, before and after treatments to thermally inactivate Rb$^+$ occlusion using anti-Leu$^{115}$–Gln$^{282}$ near the C-terminal end of the M5/M6 fragment, and, in addition, measurements of the Rb$^+$ occlusion levels. In 19-kDa membranes derived from pig kidney, the fragment Gln$^{737}$–Arg$^{830}$ migrates on Tricine gels with an apparent molecular weight of 8 kDa (8). In Fig. 9A, the membranes were suspended in a medium containing 10 mM RbCl or Tris-HCl and incubated at 37 °C for 10 min. Incubation in the Tris-HCl medium was indeed associated with release of the M5/M6 fragment into the medium. However, dissociation of the fragment did not appear

### Table III

**Kinetic parameters for inactivation of Rb$^+$ occlusion in the presence of Rb$^+$ ions**

| Temperature °C | $k_1$ ± S.E. | $k_2$ ± S.E. | $k_3$ ± S.E. | $A_1$ | $A_2$ |
|---------------|-------------|-------------|-------------|------|------|
| 45            | 0.22 ± 0.11 | 0.77 ± 0.46 | 0.04 ± 0.01 | 20.7 | 79.3 |
| 47            | 0.37 ± 0.16 | 0.68 ± 0.57 | 0.10 ± 0.03 | 24.4 | 75.6 |
| 50            | 0.32 ± 0.11 | 0.35 ± 0.24 | 0.13 ± 0.04 | 46.6 | 53.4 |
| 52            | 0.92 ± 0.14 | 100         |             |      |      |
| 55            | 1.78 ± 0.30 | 100         |             |      |      |

**Fig. 6. Protection against thermal inactivation by ouabain or ouabain plus Mg$^{2+}$ ions.** 70 µg of 19-kDa membranes were resuspended in Rb$^+$-free medium in the absence or presence of 5 mM ouabain or 5 mM ouabain plus 10 mM MgCl$_2$. Samples were pre-equilibrated at 20–22 °C for 40 min in standard medium and transferred to 37 °C for 5 min. Ice-cold reaction medium containing 5 mM RbCl plus 0.5 mM Rb$^+$ was added, and occlusion was then measured after transfer of samples and incubation at room temperature (20–22 °C) for 1 h.
A

Fig. 7. Comparison of effect of ouabain and Rb\(^+\) on protection against thermal inactivation of Rb\(^+\) occlusion in 19-kDa membranes. 19-kDa membranes were incubated at 37 °C in standard medium containing 5 mM ouabain or 0.5 or 50 mM RbCl as indicated. At the indicated times, aliquots were withdrawn, and Rb\(^+\) occlusion was measured as described for Fig. 6.

to be quantitative, with a significant proportion remaining in the membrane fraction. Rb\(^+\) occlusion was intact in the presence of RbCl, but was nearly fully inactivated in the Tris-HCl medium. This lack of full dissociation of the fragment and the nearly complete inhibition of Rb\(^+\) occlusion are significant discrepancies from the findings of the previous work (2), in which quantitative dissociation of the fragment and only ~50% inhibition of Rb\(^+\) occlusion were reported. Therefore, we have examined the quantitative aspects in more detail. Fig. 10 (A and B) presents immunoblots of the membranes and supernatant after different times of incubation at 37 °C in the Tris-HCl medium and quantification of the amount of M5/M6 fragment remaining in the membrane together with Rb\(^+\) occlusion levels. A preliminary experiment showed that the scanned signal from the M5/M6 fragment was proportional to the amount of protein applied to the gel. Evidently, no more than ~50% of the fragment was released at the longest incubation times, although Rb\(^+\) occlusion was completely inactivated. Furthermore, inhibition of Rb\(^+\) occlusion preceded the release of the fragment. Within 1 min of incubation at 37 °C, when Rb\(^+\) occlusion was ~30% inhibited, there was no detectable loss of the fragment. Fig. 9B presents the results of a second type of experiment in which Rb\(^+\) occlusion was inhibited by incubation with Ca\(^{2+}\) ions at 37 °C as described before (10). Under this condition, we did not detect dissociation of the M5/M6 fragment, although Rb\(^+\) occlusion was fully inhibited. This result constitutes a further difference from the results of Lutsenko and Kaplan (2), who stated that Ca\(^{2+}\) ions were unable to prevent dissociation of the M5/M6 fragment. In summary, the experiments confirm dissociation of the M5/M6 fragment, but show that this phenomenon is not a prerequisite for thermal inactivation of Rb\(^+\) occlusion.

Experiments similar to those in Figs. 9 and 10 (data not shown), using antibodies raised against the other fragments of 19-kDa membranes (see “Experimental Procedures”), showed no significant dissociation of the M1/M2, M3/M4, and M7/M10 fragments of the \(\alpha\)-subunit; the 16-kDa N-terminal fragment of the \(\beta\)-subunit; or the \(\gamma\)-subunit. About 10–20% of the 50-kDa extracellular fragment of the \(\beta\)-subunit was released into the medium. The time course was even slower than that for release of the M5/M6 fragments, and it occurred also in the presence of Ca\(^{2+}\) ions. Thus, there is no direct connection between release of the two fragments. The lack of dissociation of the M3/M4 fragment is not in agreement with a recent claim that 60–70% of this fragment dissociates together with the M5/M6 fragment (26).

**DISCUSSION**

**Kinetic Analysis of Thermal Inactivation and Protection by Occluded Cations and Ouabain**—The biphasic kinetics of thermal inactivation of Rb\(^+\) occlusion (Fig. 1 and Table I, 25–35 °C) led us to analyze the data by the two-step model for irreversible denaturation (27), corresponding to stages in the disorganization of the fragments (Fig. 11). For the temperature range 25–35 °C and in the absence of Rb\(^+\) ions, the good fit of the biphasic curves in Fig. 1 to Equation 4 in the “Appendix” provides one line of evidence consistent with this mechanism. A second line of evidence compatible with the model is the reversible inactivation observed at 25 °C (Fig. 3). By applying the fitted rate constants at 25 °C (Table I) to Equations 4 and 5 in the “Appendix,” the estimated fractions of N, U, and I after 30 min are 75, 17, and 8%, respectively. The predicted initial and final levels of occlusion in the reversibility experiment are 75 and 93% of control levels, respectively. These values are not inconsistent with the data in Fig. 3. Large positive values of both \(\Delta H^\circ\) and \(\Delta S^\circ\) for \(k_1\) and \(k_2\) are typical of entropy-driven...
Thermal Inactivation of Proteolized Na,K-ATPase

Fig. 9. Immunoblot analysis of dissociation of M5/M6 fragment at 37 °C in absence of Rb’ ions (A) and in presence of Ca2+ ions (B). For details of procedures and immunoblots using anti-Leu815–Gln828, see “Experimental Procedures.” The asterisks depict the positions of the M5/M6 fragment. Lane M, molecular mass markers.

Fig. 10. Time course of dissociation of M5/M6 fragment and inactivation of Rb’ occlusion. For details, see “Experimental Procedures” and the legend to Fig. 9.

Calculated equilibrium constant \( k_{2}/k_{1} \) using the values of \( k_{1} \) and \( k_{2} \) in Table I (ratios are 0.25, 1.55, and 3.16 at 25, 30, and 35 °C, respectively). \( \Delta H^{0} \) and \( \Delta S^{0} \) derived from a van’t Hoff plot are 46 kcal mol \(^{-1} \) and 153 cal K \(^{-1} \) mol \(^{-1} \), respectively. The two-step mechanism seems to relax into a one-step inactivation mechanism (N \( \rightarrow \) I) at \(-37 \) °C and higher temperatures since the kinetics were fitted adequately by a single exponential decay. Conversion of the two-step to a one-step mechanism implies that the U form is present only in negligible amounts. Lack of reversibility of inactivation at 37 °C (Fig. 3) is consistent with absence of the U form. Disappearance of the U form as a significant intermediate could occur if \( k_{3} \) rose and \( k_{2} \) fell sharply over a narrow temperature range, implying that the disorganization process is highly cooperative.

To explain biphasic kinetics, more complex schemes, invoking the existence of two populations of enzyme molecules or two pools of occluded Rb’ ions within the same molecule (26), might be considered. Such mechanisms would have to assume both different rates of thermal inactivation and interconversion between the pools since the amplitudes of the phases are strongly affected by temperature (Table I). Apart from their additional complexity, neither of these mechanisms predicts reversible inactivation (Fig. 3), and they are therefore unlikely explanations of the results described here.

Occluded cations protect strongly against thermal inactivation, as shown in Figs. 4 and 5. In the presence of Rb’ ions, the two-step Lumry-Eyring mechanism fits the data between 45 and 50 °C, but at \( 52 \) °C and higher temperatures, it appears to break down, and a single exponential decay suffices. It is of interest to compare fitted rate constants (\( k_{1}, k_{2}, \) and \( k_{3} \)) without and with Rb’ ions (Tables I and III), although the comparisons are qualitative or only semiquantitative due to the limits on accuracy of the data and lack of overlap of the temperature ranges. A direct experimental comparison was possible for \( k_{1} \) at \( 45 \) °C (1.02 ± 0.23 min \(^{-1} \) without Rb’ ions versus 0.22 ± 0.11 min \(^{-1} \) with Rb’ ions). For \( k_{1} \) at other temperatures and \( k_{2} \) and \( k_{3} \), the comparison is indirect due to the lack of overlap of accessible temperatures (25–35 °C without Rb’ ions and 45–50 °C with Rb’ ions). Inspection of the values of \( k_{1}, k_{2}, \) and \( k_{3} \) at \( 45 \) °C with Rb’ ions (Table III) and at \( 35 \) °C without Rb’ ions (Table I), together with the observed tendency of \( k_{1} \) and \( k_{3} \) to rise and of \( k_{2} \) to fall as temperature rises, makes it clear that Rb’ ions must lower \( k_{1} \) and \( k_{3} \) and raise \( k_{2} \). An effect of Rb’ ions to suppress \( k_{1} \) is not surprising. However, an effect of Rb’ ions on \( k_{2} \) and \( k_{3} \) is paradoxical because the implication is that Rb’ ions bind to the U form, whereas the two-step model assumes that only the N form is able to occlude Rb’ ions. To resolve this paradox, we propose that the product of the first

disorganization processes, whereas a value of \( \Delta H^{0} \) close to zero and a strongly negative \( \Delta S^{0} \) provide a strong indication that \( k_{2} \) reflects a reorganization process (Table II). That the N \( \rightarrow \) U equilibrium involves disorganization and reorganization can also be inferred from estimates of \( \Delta H^{0} \) and \( \Delta S^{0} \) based on the
FIG. 11. Schematic model with stages of thermal inactivation of Rb
occlusion. The model depicts the stages of thermal disorganization of a four-helix bundle. N, gates are closed, and two ions are occluded. Interactions between extra-membrane tails are intact. U, gates are open, and occluded cations dissociate. Interactions between extra-membrane tails are intact. I, the complex is fully disorganized. After thermal inactivation, the M5/M6 fragment dissociates.

The kinetics of thermal inactivation of Na,K-ATPase, which is strongly protected by K+ ions (15, 16), may also proceed by disorganization of transmembrane segments. Indeed, in experiments using intact renal microsomes, we have demonstrated directly a change in transmembrane topology in the C-terminal region of the α-subunit accompanying thermal inactivation of Na,K-ATPase activity or Rb+ occlusion (32). Rb+ ions protected strongly against both inactivation of function and disorganization of transmembrane segments (32).

The experiments in Figs. 9 and 10 confirm the observation of selective dissociation of the M5/M6 fragment, consistent with a central role of transmembrane segments M5 and M6 in cation occlusion (2, 33). However, differences in the experimental findings from those reported by Lutsenko and Kaplan (2) affect the interpretation of this phenomenon. In our experiments, only ~50% of the M5/M6 fragment was released when occlusion was fully inactivated, and inactivation of occlusion preceded release of the fragment. In addition, no release of the fragment accompanied thermal inactivation in the presence of Ca2+ ions. A possible explanation of these differences is that detection is more sensitive with use of the antibody compared with N-terminal sequencing of the fragment as used by Lutsenko and Kaplan (2). At 25 °C, the condition for reversible inactivation, the M5/M6 fragment was not released (data not shown), demonstrating also that dissociation is not necessary for thermal inactivation.

The central conclusion from the results in Figs. 9 and 10 is that dissociation of the M5/M6 fragment is not the direct cause of loss of Rb+ occlusion, but rather thermal inactivation disrupts protein-protein interactions, inactivating the Rb+ occlusion, and then the M5/M6 fragment is released into the medium (Fig. 11). A corollary is that dissociation of the M5/M6 fragment cannot be taken as evidence for a “piston-like” movement in the normal cation transport cycle (2, 33) because the fragment is dissociating from an inactive disorganized form of the enzyme. A finding that the M5/M6 fragment of H,K-ATPase is released from the membrane after washing with carbonate at pH 10 (34) is also suggestive of an alkali-dependent disorganization of the fragments prior to dissociation.

Interactions between the M5/M6 and M7/M10 Fragments—Experiments using 19-kDa membranes have shown that both step of thermal inactivation, U, is only partially disorganized and that Rb+ ions are still able to bind with a low affinity. Due to the rapid dissociation of the Rb+ ions, occlusion of Rb+ is not detectable, but the low affinity binding of the Rb+ ions elevates $k_a$ and suppresses $k_d$. The second step (U → I) leads to a more disorganized conformation with no Rb+ recognition. These concepts are not incompatible with the schematic structural model in Fig. 11.

Protection against thermal inactivation by ouabain suggests that ouabain and Rb+ ions induce similar stabilizing interactions. The combination of ouabain and Rb+ produces a further stabilization (Fig. 7). Ouabain reduces the rate of dissociation of occluded Rb+ ions (11, 30), and a part of the additional stabilization could be due to this effect, but the finding that ouabain confers extra stabilization even at very high concentration of Rb+ ions (Fig. 7) shows that it must induce additional interactions. The latter conclusion might explain our finding that a combination of Rb+ ions and ouabain was required to stabilize a detergent-solubilized complex of fragments of 19-kDa membranes (11).

Model for Thermal Inactivation of Occlusion and Dissociation of the M5/M6 Fragment—The kinetics of thermal inactivation (Figs. 1 and 3 and Table I–III) and proteolysis experiments (Fig. 8) provide complementary evidence for two stages of disorganization of the occlusion cage. These stages and the subsequent dissociation of the M5/M6 fragment are depicted in the schematic model of transmembrane helices in Fig. 11. The model also indicates that, at 37 °C and higher temperatures without Rb+ ions (or at 52 °C or higher temperatures with Rb+ ions), the intermediate U form is not significant, and a one-step mechanism of thermal inactivation suffices. The reversible step (N ↔ U) appears to involve disorganization within the membrane domain since the extra-membrane loops and tails are inaccessible to proteases under conditions of reversible inactivation (Fig. 8), suggesting that the secondary structure and interactions of these segments are largely intact. An important implication is that barriers or “gates” to dissociation of occluded cations are located within transmembrane segments and are broken in this first step. The U form might be thought of as a “molten globule,” in which the tertiary structure is disrupted, but the overall folding and secondary structure are largely native (31). The subsequent irreversible step (U → I) involves more extensive disorganization in which the interactions between extra-membrane tails and loops and their secondary structure are disrupted, and so these segments become accessible to proteases, as we have described previously (10, 13). The strong protection of occluded Rb+ and other cations against the disorganization depicted in Fig. 11 implies that the stabilizing effects are not restricted to the occlusion cage, but occur both within the membrane domain and outside the membrane, i.e. these are global effects. The latter conclusion is consistent with results of proteolysis experiments using 19-kDa membranes that show that occluded cations induce substantial structural changes in fragments of both α- and β-subunits (9, 10).
occluded cations (K\(^+\), Na\(^+\), and congeners) and ouabain protect against thermal inactivation of Rb\(^+\) occlusion (described here and see also Refs. 1 and 10); they protect the M7/M10 fragment against digestion by proteases (7–9); and they both also protect against dissociation of the M5/M6 fragment (2). These findings provide suggestive, if indirect, evidence that both Rb\(^+\) ions and ouabain induce stabilizing interactions between the M5/M6 and M7/M10 fragments and that thermal inactivation results from disruption of these interactions. Further indirect evidence for interactions between the M5/M6 and M7/M10 fragments comes from observations that dissociation of the M5/M6 fragment results in exposure of cysteine residues in the M7/M10 fragment to selective chemical modification (2, 17).

We have recently obtained direct evidence for interactions between the M5/M6 and M7/M10 fragments of 19-kDa membranes based on covalent cross-linking. This work provides a stronger indication that interactions between the M5/M6 and M7/M10 fragments are essential for maintaining intact the cation occlusion and transport structures.

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APPENDIX

The kinetic equation used was Equation 3 under “Results.” This model leads to Equations 4–6,

\[
N = \frac{N_0}{(\lambda_2 - \lambda_3)}(1 + \lambda_3 e^{\lambda_3 t} - (1 + \lambda_3) e^{\lambda_2 t}) \tag{Eq. 4}
\]

\[
U = \frac{N_0(1 + \lambda_3)(1 + \lambda_2)}{\lambda_3(\lambda_3 - \lambda_2)}(e^{\lambda_3 t} - e^{\lambda_2 t}) \tag{Eq. 5}
\]

\[
I = \frac{N_0 k_3(1 + \lambda_3)(1 + \lambda_2)}{\lambda_3(\lambda_3 - \lambda_2)}(\lambda_3(e^{\lambda_3 t} - 1) - \lambda_2(e^{\lambda_2 t} - 1)) \tag{Eq. 6}
\]

where \(\lambda_2 = -(k_1 + k_2 + k_3) + \frac{\sqrt{D}}{2}\), \(\lambda_3 = -(k_1 + k_2 + k_3) - \frac{\sqrt{D}}{2}\), and \(D = k_1^2 + k_2^2 + k_3^2 + 2(k_1 k_2 + k_2 k_3 - k_1 k_3)\) and \(A_1 = N_0(k_1 + \lambda_2) / (\lambda_2 - \lambda_3)\) and \(A_2 = N_0(k_1 + \lambda_3) / (\lambda_3 - \lambda_2)\).

\(3\) E. Or, R. Goldshleger, A. Shainskaya, and S. J. D. Karlish, submitted for publication.

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Alla Shainskaya, Victor Nesaty and Steven J. D. Karlish

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