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Binding of Sodium Ions and Cardiotonic Steroids to Native and Selectively Trypsinized Na,K Pump, Detected by Charge Movements*

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A fluorescent dye, RH421, has been used to characterize charge movements associated with cation and cardiotonic steroid binding to Na,K-ATPase and to a specifically trypsinized preparation, so-called "19-kDa membranes." A fluorescence decrease induced by Na^+ is attributed to electrogenic binding of one Na^+ ion from the cytoplasm. The apparent affinity for Na^+ is the same in both preparations. (ATP + Na + Mg) or (P_i + Mg)-induced fluorescence signals observed with native enzyme are not observed in 19-kDa membranes, consistent with loss of ATP binding and phosphorylation. Cardiotonic steroids (CS) bind to native enzyme and 19-kDa membranes as judged by RH421 signals, fluorescence of anthroyl ouabain, and inhibition of Rb^+ occlusion. Binding affinities to both preparations are in the micromolar range, and binding is prevented by the presence of Na^+ or K^+. The kinetics of glycone binding and dissociation are identical in both preparations, but aglycones bind and dissociate about 6-fold faster to 19-kDa membranes. Binding of Na^+ and cardiotonic steroids is inactivated upon heating or extensive Pronase digestion of 19-kDa membranes. This suggests that cation and CS binding depend on the structural integrity of a complex of the proteolytic fragments, and that sites for both cations or CS consist of ligating groups located on more than one fragments of 19-kDa membranes.

An understanding of active ion transport by the Na,K pump requires knowledge on the cyclic processes involved in ATP hydrolysis and the vectorial process of cation translocation. The operation of the Na,K pump involves a sequence of conformational transitions, phosphorylation and dephosphorylation, ion binding, and release reactions, referred to as the Post-Albers reaction cycle (Fig. 1) (Glynn, 1985; Jørgensen and Andersen, 1988; Läuger, 1991). In the absence of detailed knowledge of the molecular structure of the protein, particularly that of the functional sites, there is a need to develop methods of specific structural modifications in order to correlate such changes with detailed functional information. At the level of the genes, one can create point mutations, truncated forms, and chimeric molecules, while at the level of the protein one can use proteolytic enzymes to produce selectively digested proteins and chemical modification of individual residues.

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The work in this paper utilizes renal Na,K-ATPase selectively and extensively digested with trypsin (Karlish *et al.*, 1990; Capasso *et al.*, 1992). The digestion removes about 50% of protein and leaves in the membrane a well defined trypsinized enzyme containing a 19-kDa peptide and three smaller peptides (apparent molecular mass 8–11.7 kDa) derived from the α-chain. The 19-kDa fragment begins at residue 831 and extends to the C terminus and contains proposed transmembrane segments 6–10 (or 6–8), while the three smaller fragments contain the proposed transmembrane segments 1–2, 3–4, and 5–6, respectively (see Capasso *et al.* (1992) and Karlish *et al.* (1993)). The β-chain is intact or is partially cut to fragments of about 50 and 16 kDa. In these so-called "19-kDa membranes," the capacity for Rb^+ and Na^+ occlusion are fully preserved, but ATPase activity and all ATP-dependent functions are lost (Karlish *et al.*, 1990). A second preparation is prepared by digestion with the nonspecific proteases Pronase and proteinase K, which remove up to 70% of the protein, including most of the β-chain, while the 19-kDa fragment or a slightly shorter 18.5-kDa fragment remains, and again, Rb^+ occlusion is unaffected (Capasso *et al.*, 1992).

The peptide fragments of the α-chain, remaining associated in the 19-kDa membranes, consist essentially of the transmembrane segments and short extracellular loops, while the cytoplasmic domains with nucleotide-binding and phosphorylation site are largely removed. The cation binding sites are suggested to be located within the membrane domain, essential parts being contributed by the 19-kDa fragment and other transmembrane segments of the smaller fragments (Karlish *et al.*, 1990; Goldshleger *et al.*, 1992). Binding of CS[1] to 19-kDa membranes has not been characterized. The membranes contain the fragment including the proposed transmembrane segments 1–2, which is thought to be essential for binding of CS. Mutagenesis studies strongly indicate that an essential part of the ouabain binding site is located between the transmembrane segment 1 and 2 of the α-subunit (Lingrel *et al.*, 1991), and there is also evidence that Cys-104 and Phe-108, presumed to be located in the first transmembrane domain, are also involved in binding of CS (Canessa *et al.*, 1993; Schultheis *et al.*, 1993).

The fluorescent dye RH 421 is a convenient and powerful tool for characterization of ion binding and translocation (Bühler *et al.*, 1991; Läuger, 1991). The dye responds to changes of electric charge within the membrane dielectric and is able to detect charge movements associated with cation binding and dissociation at extracellular and cytoplasmic surfaces of the protein and is also able to detect conformational changes (Bühler *et al.*, 1991; Stürmer *et al.*, 1991; Heyse *et al.*, 1994). Recently, RH 421

[1] The abbreviations used are: CS, cardiac steroids; AO, anthroyl ouabain; TPP^+, tetraphenyl phosphonium; *E*, enzyme.

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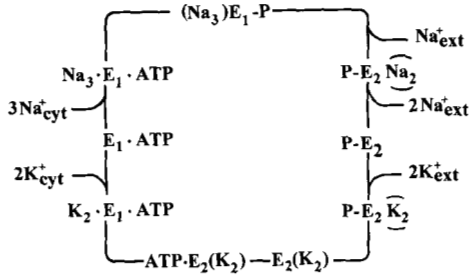

FIG. 1. **Post-Albers reaction scheme of the Na,K pump under physiological conditions.** E_1 and E_2 are conformations of the enzyme with the ion binding sites exposed to the cytoplasm and the extracellular medium, respectively. In the occluded states $(Na_3)E_1$-P, $E_2(K_2)$, and ATP·$E_2(K_2)$, the bound ions are unable to exchange with ions in either aqueous phases.

has also been shown to be useful for characterization of binding of CS because of its ability to monitor the associated cation binding (Stürmer and Apell, 1992). CS are specific inhibitors of the Na,K pump and are known to bind and inhibit the enzyme in specific conformations (Forbush, 1983). Mg^{2+} ions are a necessary prerequisite for the binding of cardiotonic steroids under all conditions, and monovalent cation Na^+ and K^+ are antagonists.

The present paper utilizes the styryl dye RH 421 in order to compare properties of the native enzyme with those of the 19-kDa membranes with respect to the binding of Na^+ and K^+ ions and cardiac steroids and the organization of and interaction between the binding sites.

EXPERIMENTAL PROCEDURES

Materials

Sodium dodecyl sulfate (SDS) was obtained from Pierce Chemical Co. For SDS-polyacrylamide gel electrophoresis, all reagents were of electrophoresis grade and were from Serva (Heidelberg, Germany). Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, NADH, ATP (disodium salt, highest available quality), and Pronase (from *Streptomyces griseus*) were from Boehringer, Mannheim, Germany. Trypsin (bovine pancreas), soybean trypsin inhibitor, thioglycolate, SDS molecular weight markers, and all cardiotonic steroids were purchased from Sigma, München, Germany. Tetraphenyl phosphonium (TPP^+) was from Merck, Darmstadt, Germany. RH 421 was from Molecular Probes, Eugene, OR. Dye purity was checked by thin-layer chromatography. All other reagents were analytical grade.

Methods

Enzyme Preparation—Na,K-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jørgensen (1974). Specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay. Protein concentration was assessed by the Lowry method, using bovine serum albumin as a standard. For most preparations, the specific activity was in the range between 1800 and 2200 μM P_i/mg of protein/h at 37 °C.

Treatment with Trypsin and Pronase—19-kDa membranes were essentially prepared according to the procedure of Capasso *et al.* (1992). Prior to the treatment, the enzyme was dialyzed overnight at 0 °C against 1000 volumes of a solution containing 25 mM histidine, 1 mM EDTA, pH 7.0, to remove all divalent cations. Subsequently, the Na,K-ATPase (3 mg/ml) was incubated for 1 h at 37 °C with trypsin (1:15 (w/w) with respect to Na,K-ATPase) in a buffer containing 10 mM Tris, 10 mM KCl, and 1 mM EDTA, pH 8.5. Thereafter, tryptic inhibitor was added (1:1, w/w) to the suspension and incubated for 10 min. After a 5-fold dilution with 25 mM imidazole (pH 7.5), 1 mM EDTA, 2 mM KCl, the membranes were centrifuged at 200,000 × *g* for 1 h. The pellet was washed in the same buffer, centrifuged, and finally resuspended in this medium to obtain a final concentration of about 1.5 mg/ml. Digestion with Pronase was adapted from Capasso *et al.* (1992). In medium containing 25 mM imidazole, pH 7.5, 1 mM EDTA, and 5 mM KCl, 19-kDa membranes were incubated with Pronase at 15 °C varying the ratio between Pronase and 19-kDa membranes from 0.33:1 to 1.25:1 (w/w). To follow the time course of the digestion, aliquots were taken at intervals and diluted 10-fold with ice-cold imidazole/EDTA/KCl buffer containing 0.5 mM phenylmethylsulfonyl fluoride to stop the reaction. Centrifugation and resuspension was performed as described for the 19-kDa membranes.

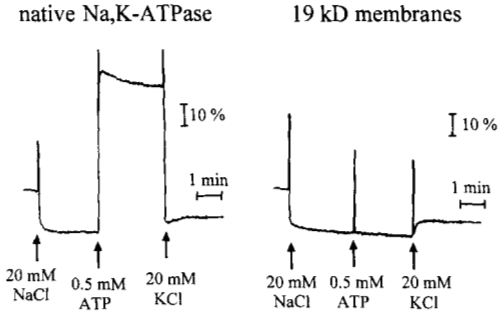

FIG. 2. **Comparison of substrate-induced partial reaction of native Na,K-ATPase in membrane fragments and 19-kDa membranes detected by the electric field-sensitive fluorescence dye RH 421.** Membranes were incubated in buffer containing 200 nM RH 421, 30 mM imidazole, 1 mM EDTA, and 5 mM $MgSO_4$ (pH 7.2). The fluorescence intensity was recorded, while 20 mM NaCl, 0.5 mM ATP, and 20 mM KCl were added successively to both membrane preparations. Temperature was 16 °C.

Fluorescence Measurements—These measurements were carried out as described previously (Bühler *et al.*, 1991) in a Perkin-Elmer model 650-40 fluorescence spectrophotometer. The thermostatically regulated cell holder was equipped with a magnetic stirrer. For experiments with RH 421, the excitation wavelength was set to 580 nm (slit width 20 nm) and the emission wavelength to 660 nm (slit width 20 nm). For experiments with anthroyl ouabain, the excitation wavelength was set to 365 nm (slit width 10 nm) and the emission was observed at 510 nm (slit width 10 nm). Experiments were carried out at 17 °C, unless indicated otherwise.

Rubidium Occlusion Assay—We used the method described by Shani *et al.* (1987). Briefly, 19-kDa membranes were incubated in buffer containing 25 mM imidazole, 1 mM EDTA, 10 mM $MgCl_2$, and various concentrations of ouabain (0–40 μM). After 20 min RbCl (+ ^{86}Rb) was added. After a 5-min incubation, the membranes were separated from RbCl on columns of Dowex 50-X8.

RESULTS

Cation Binding and Dissociation—Figs. 2 and 3 present results of experiments to characterize RH 421 signals associated with (*a*) cytoplasmic sodium binding, (*b*) phosphorylation and extracellular sodium release, and (*c*) extracellular potassium binding and dephosphorylation. Protein (10 μg/ml) was incubated in the reaction medium until the fluorescence intensity was constant, and then aliquots of NaCl, ATP, or KCl were added as indicated.

(*a*) Addition of NaCl (20 mM) produced a fluorescence decrease of about 20% for the native enzyme and 16% for the 19-kDa membranes (Fig. 2). This difference in the signal amplitude was a reproducible feature. Since the occlusion capacity is the same for both preparations (Karlish *et al.*, 1990), the difference cannot be due to different amounts of bound Na^+ ions. A possible explanation will be presented below. Titration curves for Na^+ ions, constructed by measuring the amplitude of the signal at different sodium concentrations, led to estimates of intrinsic dissociation constants of 8 mM in the presence of Mg^{2+} and 0.7 mM in the absence of Mg^{2+}, for both preparations (Fig. 3).

(*b*) Addition of 0.5 mM ATP to the native enzyme, following addition of Na^+ ions (Fig. 2), triggers the sequence of sodium occlusion, protein phosphorylation, the conformational transition $E_1 \rightarrow E_2$, deocclusion of the sodium ions, and release to the extracellular side. The large fluorescence increase, about 80%, associated with these events, is associated with release of up to 3 Na^+ ions from their binding sites at the extracellular face (Stürmer *et al.*, 1991; Heyse *et al.*, 1994). Addition of ATP to 19-kDa membranes does not change the RH 421 fluorescence

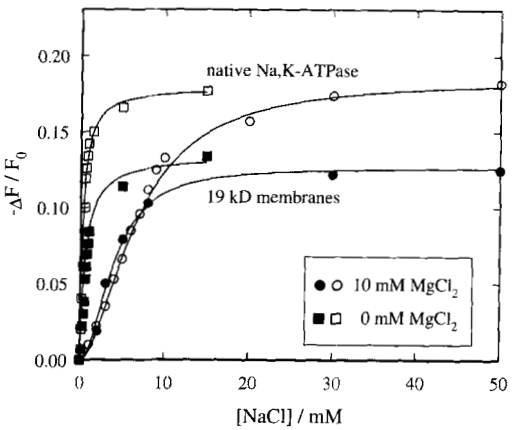

FIG. 3. **Titration of Na^+-induced RH 421 fluorescence change in the absence and presence of 10 mM Mg^{2+} for native enzyme and 19-kDa membranes.** Buffer composition was 300 mM choline chloride, 25 mM histidine, 0.5 mM EDTA, pH 7.2, at 16 °C. The fluorescence changes are attributed to electrogenic Na^+ binding to the cytoplasmic binding sites (*open symbols*, native enzyme; *solid symbols*, 19-kDa membranes). Apparent binding constants are the same for native and 19-kDa membranes: 0.7 mM (0 Mg^{2+}; ■, □) and 8 mM (10 Mg^{2+}; ●, ○).

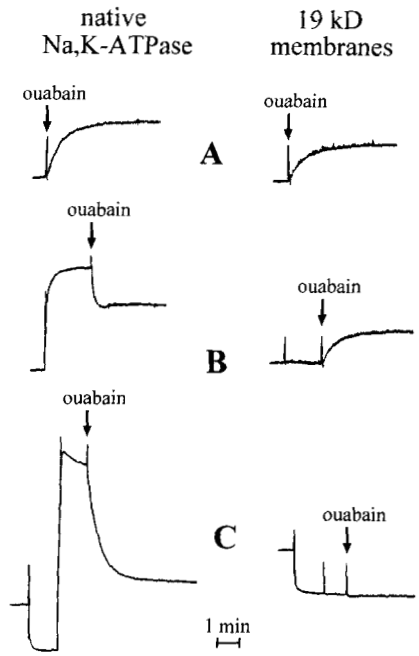

FIG. 4. **Binding of ouabain to native enzyme and 19-kDa membranes detected by changes of the RH 421 fluorescence.** Experimental conditions as in Fig. 2. The fluorescence is plotted in arbitrary units. *A*, addition of 50 μM ouabain in the absence of Na^+, ATP, or P_i; *B*, successive addition of 50 μM P_i and 50 μM ouabain; *C*, successive addition of 20 mM NaCl, 0.5 mM ATP, and 50 μM ouabain.

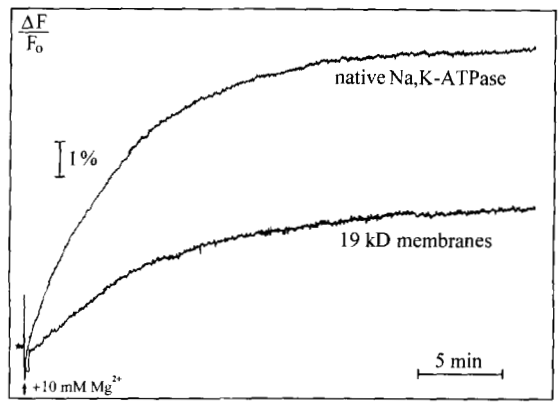

FIG. 5. **Fluorescence increase detected by binding of anthroyl ouabain.** Native enzyme or 19-kDa membranes were equilibrated in buffer containing 25 mM histidine, 0.5 mM EDTA, and 10 μM anthroyl ouabain. At time zero, 10 mM $MgCl_2$ were added to trigger inhibitor binding. Increase of the fluorescence intensity corresponds to an binding to the enzyme (Fortes, 1986). F_o is the fluorescence level prior to addition of $MgCl_2$.

signal (Fig. 2), consistent with the observation that all ATP-dependent functions are absent in 19-kDa membranes (Karlish *et al.*, 1990).

(*c*) Addition of 20 mM KCl to native enzyme following addition of ATP leads to a fluorescence decrease of about 60% (Fig. 2). The sequence of events involves potassium binding to the phosphorylated E_2 states at extracellular sites, occlusion of K^+ ions and dephosphorylation of the protein (Fig. 2). Following addition of K^+ ions, the enzyme will be mainly in states $E_2(K_2)$ and P-$E_1(Na_3)$ in the conditions of this experiment (Heyse *et al.*, 1994), and the fluorescence decrease is caused most likely by the uptake of 2 (or 3) positive charges into the protein dielectric when it forms the states $E_2(K_2)$ and P-$E_1(Na_3)$. Addition of 20 mM KCl to the 19-kDa membranes increases the RH 421 fluorescence by about 8% (Fig. 2) and is explained by binding of K^+ ions, forming a mixture of states $E_1(Na_3)$ and $E_2(K_2)$, according to the relative affinities for Na^+ and K^+ ions.

Addition of K^+ ions alone to either native enzyme or 19-kDa membranes produces no significant change in RH 421 fluorescence (not shown).

Binding of Cardiotonic Steroids to Native Enzyme and 19-kDa Membranes—Fig. 4 shows the RH 421 signals accompanying binding of CS to the Na,K pump in three different conditions: binding to the enzyme in the absence of Na^+, K^+, ATP, and P_i (Fig. 4*A*), binding to the P_i-phosphorylated enzyme in the absence of Na^+ and K^+ (Fig. 4*B*), and binding to the enzyme phosphorylated by ATP in the presence of 20 mM Na^+ (Fig. 4*C*). In all three cases 5 mM Mg^{2+} was present in the buffer. The molecular origin of the ouabain-induced fluorescence signals in Fig. 4 is not understood in detail (see "Discussion"). The main purpose of the experiment is to demonstrate the fact of specific ouabain binding to both native and trysinized enzyme.

Addition of 50 μM ouabain to membrane fragments suspended in a buffer containing 5 mM $MgSO_4$, but no Na^+, K^+, ATP, or P_i, produced a fluorescence increase of 18% for native enzyme and 11% for 19-kDa membranes (Fig. 4*A*). The signals are caused by specific binding of ouabain, since no fluorescence increase could be detected in the absence of Mg^{2+} ions, even at ouabain concentrations as high as 1 mM, or in the presence of high concentrations of Na^+ (>200 mM) or K^+ (>50 mM), which are also known to prevent ouabain binding (Hansen, 1984). Finally, we have studied binding of anthroyl ouabain (AO) to native enzyme and 19-kDa membranes (Fig. 5). The quantum yield of fluorescence of this ouabain analog increases upon binding to the Na,K pump (Fortes, 1977, 1986). Binding of AO (10 μM) was indicated by the increase in fluorescence intensity and was triggered by addition of Mg^{2+}. AO binds to both native and fragmented enzyme, and as expected, no change of the AO fluorescence was observed in the absence of Mg^{2+} or presence of Na^+ or K^+ ions.

Fig. 4*B* (*left*) shows the RH 421 fluorescence response upon addition of 50 mM P_i and 50 μM ouabain to native enzyme equilibrated in a buffer containing 5 mM $MgSO_4$, but no Na^+ or K^+ ions. Addition of P_i induced a fluorescence increase, indicating a release of positive (or uptake of negative) charge (Bühler *et al.*, 1991). Subsequent addition of ouabain decreased the RH 421 fluorescence, indicating a reverse charge movement upon ouabain binding. Addition of P_i to 19-kDa membranes does not

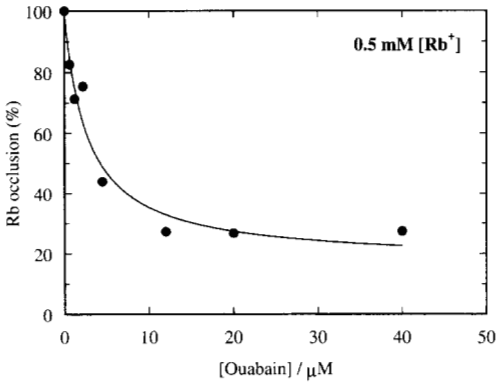

FIG. 6. **Inhibition of Rb^+ occlusion in 19-kDa membranes by ouabain.** Aliquots of the protein were incubated in given concentrations of ouabain. Subsequently the ability to occlude ^{86}Rb was measured. Buffer composition was 25 mM imidazole, 1 mM EDTA, and 10 mM $MgCl_2$. Data were normalized to the level of occlusion of Rb^+ in the absence of ouabain. Apparent K_I of ouabain was 2.3 μM in the presence of 0.5 mM RbCl.

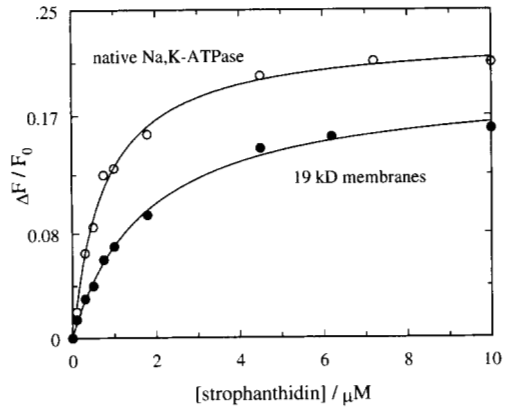

FIG. 7. **Comparison of the binding affinities of strophanthidin for native Na,K-ATPase and 19-kDa membranes.** For each concentration of strophanthidin, the inhibitor-induced fluorescence change was determined. Buffer composition was 5 mM histidine, 5 mM $MgCl_2$, pH 7.2, 10–15 mg of protein, at 16 °C.

change the RH 421 fluorescence (Fig. 4*B*, *right*), consistent with the absence of ATP binding and phosphorylation sites (Karlish *et al.*, 1990). Subsequent addition of ouabain to the 19-kDa membranes resulted in an increase of the RH 421 fluorescence. This change was not observed in the absence of Mg^{2+} ions or presence of high concentrations of Na^+ or K^+ ions.

Fig. 4*C* depicts the RH 421 signals accompanying addition of 50 μM ouabain after addition of Na^+ ions and ATP. Addition of 20 mM Na^+ and then 0.5 mM ATP to native enzyme induces the standard response described in Fig. 2. Subsequent addition of ouabain produces a fluorescence decrease of approximately 50%. This fluorescence decrease corresponds to an uptake of positive charge to the enzyme and has been attributed to occlusion of 2 Na^+ ions upon binding of CS (Stürmer and Apell, 1992). Note that the level of fluorescence after addition of ouabain is close to that obtained by CS binding in the absence of P_i and ATP (Fig. 4*A*). Fluorescence does not change when the same experiment is performed using 19-kDa membranes, a finding consistent with that of previous control experiments.

Another way of demonstrating binding of ouabain on 19-kDa membranes, was to look at effects of the CS on Rb^+ occlusion. In the experiment in Fig. 6, aliquots of 19-kDa membranes were equilibrated with various concentrations of ouabain in a buffer containing Mg^{2+} but no Na^+, K^+ ions, or P_i, and ^{86}Rb occlusion was measured at 0.1 mM, 0.5 mM, and 5 mM Rb. Ouabain inhibited Rb^+ binding to 19-kDa membranes, and at each Rb^+ concentration the equilibrium inhibition constant, K_I, of ouabain was determined, giving apparent values between 1.1 and 2.3 μM. A comparable experiment with native enzyme at 0.5 mM Rb gave a K_I value of 1.7 μM (not shown).

Comparison of Binding of Glycones and Aglycones—Yoda and Yoda (1973) compared different binding properties of glycones and aglycones and inferred that the steroid and sugar moieties bind to different sites on the protein. It was of interest to determine whether the differences are preserved in 19-kDa membranes. RH 421 fluorescence signals were used to compare the equilibrium binding of strophanthidin (Fig. 7) and kinetics of binding ouabain and its aglycone ouabagenin (Fig. 8, *A–D*) and that of strophanthidin (Fig. 9).

The binding affinities for strophanthidin of native enzyme and 19-kDa membranes were compared (Fig. 7). The aglycone strophanthidin was chosen for convenience, because it binds significantly faster to the enzyme than does ouabain. The concentration dependence could be described by hyperbolic curves with half-maximal fluorescence changes at 0.75 μM strophanthidin for native enzyme and 1.25 μM for 19-kDa membranes.

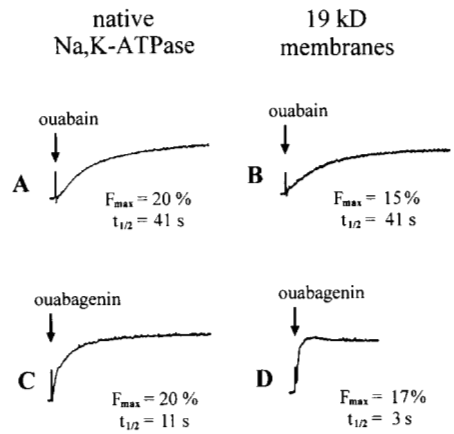

FIG. 8. **Time course of the RH 421 fluorescence change induced by addition of different cardiac glycosides.** *A* and *B*, 50 μM ouabain; *C* and *D*, 50 μM ouabagenin. Native Na,K-ATPase or 19-kDa membranes were equilibrated in buffer containing 200 nM RH 421, 25 mM histidine, 0.5 mM EDTA, and 10 mM $MgCl_2$, pH 7.2, at 16 °C. From the time course of experiment the maximum fluorescence increase, F_{max}, and the saturation half-time, $t_{1/2}$, was determined as noted.

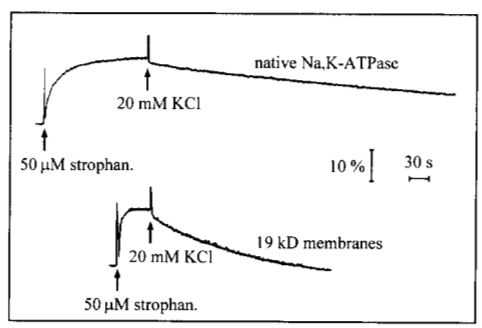

FIG. 9. **K^+-induced dissociation of strophanthidin detected by the fluorescence change of RH 421.** To native Na,K-ATPase (*upper trace*) or 19-kDa membranes (*lower trace*) in a buffer (which contained 200 nM RH 421, 25 mM histidine, 0.5 mM EDTA, and 10 mM $MgCl_2$), 50 μM strophanthidin and 20 mM KCl were added successively. In case of the native pump, the fluorescence signal was followed for 1200 s (not shown). For the rising and falling part of the fluorescence signals, the corresponding saturation half-times, $t_{1/2}$, were determined (see "Results").

The experiment in Fig. 8 (*A* and *B*) shows that upon addition of 50 μM ouabain, the RH 421 fluorescence increased 20% for the native and 15% for the 19-kDa membranes, and the time

course of both signals could be fitted approximately by a single exponential with a half-time, $t_{1/2}$, of 41 s. Ouabagenin produced fluorescence increases of 21% for the native and 17% for the 19-kDa membranes, and much faster rates of $t_{1/2}$ of 11 s for native membranes and $t_{1/2}$ of 2 s for the 19-kDa membranes (Fig. 8, *C* and *D*). Thus ouabagenin binds even faster to 19-kDa membranes than to native enzyme. Addition of the aglycone strophanthidin to native enzyme and 19-kDa membranes (Fig. 9) resulted in RH 421 fluorescence increases with time courses ($t_{1/2}$ of 12 and 2 s, respectively), similar to those observed with aglycone ouabagenin (Fig. 8). Subsequent addition of 20 mM KCl induced dissociation of the aglycone and reversal of the signal change. The affinity for strophanthidin binding was approximately the same in both preparations (Fig. 7), and thus a 6-fold faster rate of association of strophanthidin to 19-kDa membranes should be matched by a faster rate of dissociation. In accordance with this expectation, the decay half-times of the fluorescence decrease were 400 and 63 s, respectively (Fig. 9). Similar results were obtained for both preparations when strophanthidin dissociation was induced by the addition of 100 mM NaCl (not shown).

Inactivation of Cation and Cardiotonic Steroid Binding—In view of the negative binding interactions between cations and cardiotonic steroids, it was of interest to determine whether the organization of their binding domains is mutually dependent. Information has been obtained by perturbing the protein structure and observing whether the RH 421 signals reporting CS and Na^+ binding are affected in a similar or different way.

One method exploits thermal inactivation of 19-kDa membranes. Incubation of 19-kDa membranes in the absence of Na^+ and K^+ ions causes a progressive loss of their ability to occlude cations, particularly at 37 °C (Or *et al.*, 1993). 19-kDa membranes were incubated for various times at 25 and 37 °C in a buffer lacking Na^+ or K^+ ions, aliquots were stored on ice until subjected to analysis, and then changes of the RH 421 fluorescence upon addition of 20 mM NaCl or 10 μM strophanthidin were determined (Fig. 10*A*). The change of signal, $\Delta F/F_0$, is proportional to the amount of charge bound to the enzyme, and is a direct measure of the remaining proportion of active 19-kDa membranes. The amplitudes of signals produced by Na^+ and strophanthidin binding, were reduced in parallel (Fig. 10*A*). The time course of the fluorescence signals remained constant within the accuracy of experiments (not shown).

A second approach utilizes extensive digestion by Pronase. 19-kDa membranes were incubated at 15 °C for various times in a suspension containing Pronase in amounts of 0.27–1.25:1 (w/w), and the preparations were analyzed by the RH 421 method to determine Na^+ and strophanthidin binding (Fig. 10*B*). At the lowest ratio of Pronase to 19-kDa membranes, 0.27:1, neither signal was significantly affected. The result is consistent with that in Capasso *et al.* (1992) in which, at this ratio of Pronase to 19-kDa membranes, the molar mass of the 19-kDa fragment is slightly reduced to 18.5 kDa and the β-chain is digested, but the capacity for Rb^+ occlusion is fully retained. However, digestion with higher concentrations of Pronase (1.25:1, w/w) resulted in a progressive inactivation with a parallel decrease of the ability to bind Na^+ and strophanthidin (Fig. 10*B*). For clarity, data for the intermediate ratio (0.67:1, w/w) are not included.

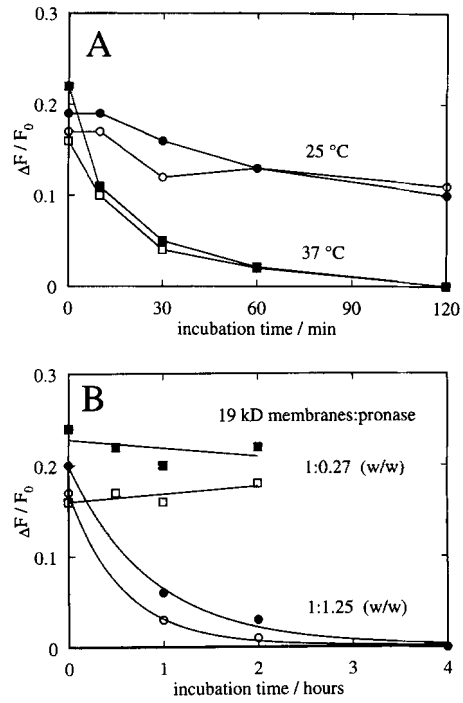

FIG. 10. **Inactivation of 19-kDa membranes.** *A*, thermal inactivation. Electrogenic Na^+ binding in an E_1-like conformation (*solid symbols*) and strophanthidin binding in an E_2-like conformation (*open symbols*) have been determined by RH 421 fluorescence changes. Experimental conditions were as given in Fig. 2*B*. Incubation was performed at 25 °C (*circles*) and 37 °C (*squares*). *B*, inactivation by Pronase. Electrogenic Na^+ binding in the E_1-like conformation (*solid symbols*) and strophanthidin binding in the E_2-like conformation (*open symbols*) have been determined by RH 421 fluorescence changes. *Squares* represent data with a ratio of 19-kDa membranes to Pronase = 1:0.27 (w/w), and *circles* represent data of 19-kDa membranes to Pronase = 1:1.25 (w/w).

DISCUSSION

Properties of RH 421 Signals on Native Enzyme and 19-kDa Membranes—The dye RH 421 responds exclusively to changes of the electric field within the membrane dielectric and not to conformational changes of the Na,K-ATPase. Therefore, it is a convenient tool to detect movements of electric charges accompanying partial reactions, such as binding of Na^+ at a cytoplasmic site, deocclusion and dissociation of Na^+ ions at the extracellular side, and inhibition of the enzyme by cardiac glycosides (Stürmer *et al.*, 1991; Stürmer and Apell, 1992; Heyse *et al.*, 1994).

It is of interest that the amplitude of the RH 421 fluorescence signals seen with 19-kDa membranes were always smaller by 20–25% than those seen with native enzyme from which they were prepared. An obvious possibility, that 20–25% of the protein had been destroyed completely by the protease treatment, can be excluded, because the capacity for occluding cations is fully maintained in 19-kDa membranes (Karlish *et al.*, 1990). It is known, on the other hand, that the electric field-induced fluorescence changes of styryl dyes are very sensitive to the local dielectric constant. Although no data are available on the distribution of the dye in the lipid phase of the membrane preparations, it can be concluded, from basic principles, that the dye molecules close to the protein molecules produce a prominent contribution to the fluorescence signal. After protease treatment, almost 50% of the protein mass is removed and the dye, which is situated close to the surface, should detect the replacement of the protein by electrolyte due to the increase of the dielectric constant. This increase leads a reduction of the fluorescence response, without changing its mechanism. The signal amplitudes would be reduced by a constant factor, but the kinetics of an electrogenic process would be recorded without distortion.

Cytoplasmic Sodium Binding—It was shown recently that it is possible to detect binding of Na^+ to a cytoplasmic ion binding site by RH 421 fluorescence change and cannot be produced by any other ion (Stürmer *et al.*, 1991; Heyse *et al.*, 1994). The site

can be assumed to be cytoplasmic, as judged both by the high affinity for Na^+, and the fact that, in high ionic strength medium, the enzyme is in an E_1 form (Skou and Esmann, 1980), a state in which Na^+ sites are oriented toward the cytoplasmic surface (Karlish and Pick, 1981). A previous proposal that Na^+ binding at the cytoplasmic surface is a charge-carrying process (Goldshlegger *et al.*, 1987) is, therefore, demonstrated directly using RH 421. Native enzyme and 19-kDa membranes show comparable fluorescence decreases, indicating import of positive charge into the membrane dielectric (Fig. 2). The equilibrium dissociation constants for Na^+ binding in the absence (0.7 mM) and presence (8 mM) of Mg^{2+} ions were found to be the same in both preparations (Fig. 3).

What is the nature of the sodium site being detected by the RH 421? It is known that binding and transport of K^+ ions or their congeners is an electroneutral process (Goldshlegger *et al.*, 1987; Läuger and Apell, 1988; Stürmer *et al.*, 1989) and does not induce a change of the RH 421 fluorescence (Stürmer *et al.*, 1991). It was proposed that the two K^+ (or two Na^+) ion binding sites contain a negatively charged group, while the third Na^+ binding site contains only neutral ligating groups (Goldshlegger *et al.*, 1987; Glynn and Karlish, 1990). A model has been proposed according to which, in the cytoplasmic orientation or E_1 conformation, the two negatively charged groups lie outside the membrane dielectric, while the third neutral sodium site is placed deeper within the dielectric (Läuger, 1991). Presumably the specific sodium binding site being detected by the RH 421 fluorescence is the neutral site. This site appears to be preserved completely intact in 19-kDa membranes since the affinity is identical to that in native enzyme.

The Mg^{2+}-induced shift of the equilibrium dissociation constant for Na^+ ions in 19-kDa membranes (Fig. 3) occurs in the absence of the ATP binding domain and enzymatic machinery, and thus, presumably, Mg^{2+} can interact with the protein directly at the ion binding sites (and not only in the form of a Mg·ATP complex). The mechanism could be a competitive occupation of the negatively charged binding sites, which are in contact with the surface of the protein (Läuger, 1991; Heyse *et al.*, 1994). Previously, we have reported that occlusion of Na^+ in 19-kDa membranes occurs with a lower apparent sodium affinity and higher degree of sigmoidicity than on native enzyme (Karlish *et al.*, 1990). Since the binding affinity for Na^+ at the neutral site appears to be unchanged (Fig. 3), one might conclude that the affinity of the two negatively charged sites is reduced in 19-kDa membranes.

Signals Accompanying Phosphorylation-Dephosphorylation—The RH 421 signals in native enzyme accompanying phosphorylation by ATP and dephosphorylation (Fig. 2, *left*) reflect deocclusion and dissociation of Na^+ ions, movement of the negative sites into the membrane dielectric, and then rebinding of K^+ ions at the extracellular surface (Stürmer *et al.*, 1991; Heyse *et al.*, 1994). These events are not seen in 19-kDa membranes (Fig. 2, *right*), because the ATP sites are absent. Similarly, the fluorescence increase accompanying phosphorylation by inorganic phosphate (Fig. 4*B*) can be observed only with the native enzyme preparation. This signal has its origin in the transition to state P-E_2, associated with movement of uncompensated negative charge in the binding sites. The reduced amplitude compared to phosphorylation by ATP in low Na^+ concentrations (Fig. 4*C*) is caused by the lower affinity of P_i compared to ATP.

Binding of Cardiotonic Steroids—The experiments in Figs. 4–9 demonstrate that the CS bind to both native enzyme and 19-kDa membranes in a specific way and produce characteristic RH 421 signals. The affinities of native enzyme and 19-kDa membranes for CS are similar (see Figs. 7 and 8). The new finding of specific CS binding on 19-kDa membranes is consistent with and supports the evidence that the proteases remove only cytoplasmic domains of the protein, while the membrane-embedded portions and extracellular loops of protein are intact (Karlish *et al.*, 1990; Capasso *et al.*, 1992).

What is the origin of the CS-induced RH 421 signals in the different conditions?

1) Addition of ouabain to either native Na,K-ATPase or 19-kDa membranes in a buffer containing Mg^{2+} ions produces a fluorescence increase (Fig. 4*A*, *left* and *right*). Important controls that indicate this binding of ouabain is specific include the following. (*a*) The RH 421 signal depends absolutely on the presence of Mg^{2+} ions, and only a negligible fluorescence change is observed in the presence of 20 mM NaCl, and (*b*) the specific binding of anthroyl ouabain (Fortes, 1986) is detected by an increase of fluorescence induced upon addition of Mg^{2+} (Fig. 5), the signals following the same kinetics with both membrane preparations.

The final level of the signal in Fig. 4*A* is comparable to that produced upon inhibition with ouabain after phosphorylation with ATP (see Fig. 4*C*, *left*). As discussed recently, the final level of fluorescence in Fig. 4*C* is thought to reflect the stabilization of a form P-$E_2(Na_2)$ with 2 Na^+ ions occluded (Stürmer and Apell, 1992). If the assumption remains valid that CS binds only to the enzyme with two cations in the binding sites, a hypothetical reaction sequence to explain the signals in Fig. 4*A* could be $E_1 \rightarrow E_2 \rightarrow E_2H_2 \rightarrow E_2H_2$-CS, in which protons bind to the sites in the sodium-free medium.

2) As seen in Fig. 4*B* (*left*), ouabain binds to native Na,K-ATPase phosphorylated by P_i and induces a fluorescence decrease, indicating an import of positive charge into the binding sites. The final level of the fluorescence corresponds to that observed upon ouabain binding in the ATP phosphorylated state (Fig. 4*C*). A proposal for the underlying reaction sequence could be again P-$E_2 \rightarrow$ P-$E_2H_2 \rightarrow$ P-E_2H_2-CS. Some experimental support for this hypothesis is provided by recent studies on the pH dependence showing that the ouabain-induced fluorescence decrease was accelerated by a factor of 2 when the pH was changed from 7.5 to 6.1 (Apell and Stürmer, 1992). Because P_i does not interact with 19-kDa membranes, the signal in Fig. 4*B* (*right*) was indistinguishable from that in the absence of P_i (Fig. 4*A*, *right*).

3) Inhibition of the Na,K pump by CS in the presence of Na^+ and ATP (see Stürmer and Apell (1992) and Heyse *et al.* (1994)). Addition of ouabain triggers the reaction sequence P-$E_2 \rightarrow$ P-$E_2Na_2 \rightarrow$ P-E_2Na_2-ouabain (Fig. 4*C*). In the case of 19-kDa membranes ouabain produces no effect onto the RH 421 fluorescence, since the presence of 20 mM Na^+ stabilized the protein in a state with 3 Na^+ ions bound, so that sodium was antagonistic to ouabain binding. As expected, addition of ATP was without effect (Fig. 2).

The interpretations of the RH 421 signals given above is supported by the finding that the presence of the hydrophobic cation TPP^+ abolishes the RH 421 fluorescence change induced by CS (data not shown). In addition, the AO fluorescence remained unchanged in the presence of TPP^+. This is compatible with the notion that the TPP^+ induces an electric field in the membrane dielectric (Stürmer *et al.*, 1991) and forces the pump out of the state P-$E_2(Na_2)$, to which the CS bind with highest affinity (Lee and Fortes, 1985; Stürmer and Apell, 1992), and into other forms to which CS are unable to bind.

Although the equilibrium binding affinities of CS to native Na,K-ATPase and 19-kDa membranes appear to be similar (Fig. 7), an interesting difference was observed in the kinetics of binding of the glycone ouabain and aglycones ouabagenin and strophanthidin (Figs. 8 and 9). Ouabain appears to bind

with identical rates to native enzyme and 19-kDa membranes (Fig. 8, *A* and *B*). Aglycones (*e.g.* ouabagenin) bind significantly faster to both native enzyme and 19-kDa membranes than do the glycones (*e.g.* ouabain) (Fig. 8). However, binding of ouabagenin to 19-kDa membranes is even faster than to native membranes (Figs. 8, *C* and *D*). Ouabain differs from ouabagenin only by the presence of the sugar residue. Thus the identical binding kinetics of ouabain to native enzyme and 19-kDa membranes indicates that the rate-limiting process is association of the sugar residue to its site, which must be intact in 19-kDa membranes. On the other hand, binding and dissociation of the aglycone strophanthidin on 19-kDa membranes are greater by approximately the same factor (6-fold) relative to those in the native enzyme (Fig. 9), although the equilibrium dissociation constant is the same for both preparations (Fig. 7). A possible explanation could be that the steroid binding domain, although intact, has been modified by the trypsin treatment, but the access of the CS to this site is facilitated.

Organization of Cation and Cardiotonic Steroid Binding Sites—What is the significance of the findings that ouabain binding and Na^+ binding are lost at an identical rate when 19-kDa membranes are inactivated by incubation at elevated temperatures (Fig. 10*A*) or by further extensive digestion with Pronase (Fig. 10*B*)? In this connection, it is important to consider the notion that proteolytic fragments of 19-kDa membranes, consisting of transmembrane segments and extracellular loops of the α-chain and intact β-chain, are arranged as a specific complex within the membrane, the cation occlusion sites consisting of ligating groups contributed by more than one segment (Capasso *et al.*, 1992; Goldshleger *et al.*, 1992). The inactivation experiments strongly support and extend this concept. Thermal inactivation could result from spatial disaggregation of the transmembrane segments, a phenomenon that is minimized at low temperatures and by occlusion of K^+ or Na^+ ions (Or *et al.*, 1993). Both CS and Na^+ binding would depend on the presence of the same structural complex of proteolytic fragments, and be destroyed when the complex is disorganized. Although the CS and Na^+ binding sites are not necessarily located on the same proteolytic fragment, the findings provide a strong hint that both sets of the sites themselves consist of ligating groups donated by more than one fragment. The example of the CS binding site illustrates the point. Site-directed mutagenesis experiments pointed to the extracellular loop between M1 and M2 as being critical for CS binding (Price and Lingrel, 1988; Lingrel *et al.*, 1991), but more recent work shows that a residue at the extracellular surface between M3 and M4 (Canessa *et al.*, 1993), and also residues in the C-terminal half of the α-chain are also important (Schultheis *et al.*, 1993; Blostein *et al.*, 1993). The implication could be that the contact sites for the cardiotonic steroid are located at several points on the extracellular surface of the protein.

Finally, the results of inactivation at high concentrations of Pronase (Fig. 10*B*) fit in well with recent work demonstrating digestion of 19-kDa membranes by trypsin in the presence of Ca^{2+} ions. Upon displacement of Rb^+ by Ca^{2+} ions, trypsin digests all the fragments of 19-kDa membranes, thus providing evidence for a Rb^+-stabilized complex of fragments (Shainskaya and Karlish, 1994). Assuming, similarly, that Pronase is able at high concentrations to split a bond which inactivates cation occlusion, this should destabilize the entire complex, making all fragments accessible to proteolysis, and so also destroy the CS binding site.

Conclusions—The work in this paper illustrates the value of combining the use of 19-kDa membranes with the RH 421-dye method. We have been able to probe the cation sites in greater detail than can be achieved with occlusion measurements, to detect and characterize binding of cardiotonic steroids, and to compare the responses to different structural perturbations.

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