

Evidence for the Existence of Two ATP-sensitive Rb⁺ Occlusion Pockets within the Transmembrane Domains of Na⁺/K⁺-ATPase*

(Received for publication, December 30, 1996, and in revised form, March 3, 1997)

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A trypsin-digested Na⁺/K⁺-ATPase that has lost ATPase activity and about half of its protein content retains an essentially intact β -subunit, the 10 transmembrane domains of the α -subunit, and the full capacity to occlude Na⁺ and Rb⁺ (a congener of K⁺). When this preparation was incubated at 37 °C in the absence of Rb⁺, it lost half of its Rb⁺ occluding capacity and two-thirds of its Na⁺ occluding capacity. Comparison of the Rb⁺ occlusion-deocclusion kinetics of the digested enzyme before and after partial inactivation indicated that (a) the affinities of the labile and the stable halves of occluded Rb⁺ were the same; (b) occlusion and deocclusion rates of the stable pool were lower than those of the labile pool; (c) ATP at a low affinity site ($K_{0.5} = 25\text{--}300\ \mu\text{M}$) increased deocclusion rate in the stable pool and occlusion rate in the labile pool; (d) Na⁺ increased Rb⁺ deocclusion rate of the sum of the two pools but not that of the stable pool; and (e) occlusion and deocclusion rates of both pools were decreased by ouabain. These findings suggest that (a) the peptide complex of the digested enzyme contains two distinct but interacting cation occlusion pockets, one occluding two Na⁺ or one Rb⁺, and the other occluding one Na⁺ or one Rb⁺; (b) this peptide complex that is devoid of the catalytic ATP site retains an allosteric ATP site; and (c) the access channels of the two pockets are regulated differently by ATP but similarly by ouabain. Analyses of the gel electrophoretic patterns of the digested enzyme and the N termini of the appropriate bands showed that inactivation of the labile occlusion pocket was accompanied by 60–70% loss of two α -fragments containing H₃-H₄ and H₅-H₆ transmembrane domains. This and the previously established interactions among the transmembrane helices of α - and β -subunits suggest that one occlusion pocket is associated with H₃-H₆ domains and that the other is located within a complex of β -subunit and two α -fragments containing H₁-H₂ and H₇-H₁₀ transmembrane domains.

Na⁺/K⁺-ATPase catalyzes the coupled active transport of Na⁺ and K⁺ across the plasma membranes of most eucaryotic cells (1). For the transmembrane movement of K⁺ during the normal cycle of the enzyme, extracellular K⁺ must pass through narrow access channels before it is occluded at the K⁺ binding sites and subsequently released inside the cell (2–5).

* This work was supported by National Institutes of Health Grant HL-36573 awarded by the National Heart, Lung, and Blood Institute, United States Public Health Service, Department of Health and Human Services. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Our previous studies on the kinetics of interaction of Rb⁺ (a congener of K⁺) with the purified Na⁺/K⁺-ATPase indicated that the width of the access channels connecting the medium to the Rb⁺ occlusion sites of the protein are allosterically regulated by Na⁺, ouabain, and ATP at a low affinity site (5). The transport of cytosolic Na⁺ to the extracellular medium also involves the occlusion of Na⁺ within the enzyme (3, 4), but the access channels of these Na⁺ occlusion sites have not been characterized at the level of the isolated purified enzyme. It is often assumed that the occlusion pockets (binding sites and access channels) for Na⁺ and K⁺ are different conformational states of the same protein domains (4, 6), although separate pathways for occlusions of Na⁺ and K⁺ have also been advocated (7).

Extensive tryptic digestion of the purified membrane-bound Na⁺/K⁺-ATPase in the presence of Rb⁺ removes about half of the enzyme protein consisting of the cytoplasmic domains of the α -subunit (8). The remaining membrane preparation, which is composed of the essentially intact β -subunit and several α -fragments containing all transmembrane domains and their extracellular loops, has no ATPase activity but retains full capacity to occlude Na⁺ and Rb⁺ (8, 9). This and related proteolytically fragmented preparations of the enzyme (10, 11) have become valuable tools for studies on the relation of structure to cation occlusion and transport. For example, it has been reported recently (12) that the selective release of one of the α -fragments (Gln⁷³⁷-Arg⁸³⁰) from the trypsin-digested preparation impairs Rb⁺ occlusion capacity, suggesting that the H₅-H₆ hairpin that is within this fragment has a key role in cation pumping by the enzyme.

The purpose of the present work was 2-fold. First, in view of the increasing use of the fragmented preparations of the enzyme for the characterization of the cation occlusion sites, we wished to use the same approach we had used with the purified intact enzyme (5) to characterize the access channels of the Rb⁺ occlusion sites in the extensively trypsin-digested enzyme. Second, we wished to assess the suggested role of H₅-H₆ transmembrane domains in the regulation of the Rb⁺ occlusion sites and their access channels.

EXPERIMENTAL PROCEDURES

Purified membrane-bound Na⁺/K⁺-ATPase of canine kidney medulla, with specific activity of 1000–1600 μmol of ATP hydrolyzed per mg of protein/h, was prepared and assayed as described before (13). This native enzyme was used to make the following two preparations. (a) The enzyme was exposed to trypsin in the presence of Rb⁺ and EDTA at pH 8.5 according to Karlish *et al.* (8) to remove large portions of the cytoplasmic domains of the α -subunit and obtain a preparation composed of an essentially intact β -subunit and four characterized fragments of α containing all 10 transmembrane helices of α (8–10). We established experimentally that after digestion less than 1% of the intact α -subunit remained. This preparation is often called the 19-kDa membrane because its cation occlusion capacity was originally thought to be due to a C-terminal fragment of α with apparent molecular mass of 19-kDa (8). Because it is now evident that more than one peptide of this preparation is involved in its functions (9, 10, 12, 14) and to avoid

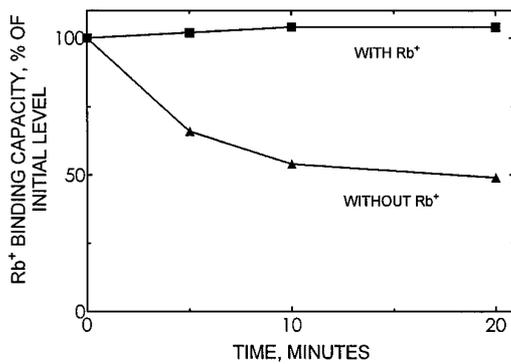


FIG. 1. **Partial inactivation of the Rb⁺ occlusion capacity of the digested enzyme.** The digested preparation was incubated at 37 °C in the absence or the presence of 10 mM Rb⁺ for the indicated times. After washing, each sample was then incubated at 4 °C for 24 h with 500 μ M ⁸⁶Rb⁺ and assayed for bound Rb⁺ as indicated under "Experimental Procedures."

confusion, we shall simply refer to this preparation as the digested enzyme. (b) The digested enzyme was suspended (1 mg/ml) in a solution containing 0.2 M sucrose and 12 mM histidine (pH 6.8) and incubated at 37 °C for 10 min. 10 volumes of the ice-cold sucrose-histidine were added, and the sample was centrifuged at 100,000 \times *g* for 1 h at 4 °C. After resuspension of the pellet and centrifugation for the second time, the pellet was then suspended in the same sucrose-histidine solution prior to use. It will be shown under "Results" that this incubation procedure causes the partial loss of cation occlusion capacity. This partially inactivated preparation will be referred to as the digested modified enzyme.

Assay of occluded Rb⁺ and experiments on the kinetics of Rb⁺ occlusion-deocclusion at 4 °C were done as we have described before (5). Occluded Na⁺ was assayed according to Karlsh *et al.* (8). When the number of experiments and standard errors are not shown, the indicated values are means of duplicate or triplicate assays. Previously described procedures were used for the labeling of the peptides with fluorescent CPM¹ (12), electrophoresis on Tricine/SDS gels (15), and N-terminal sequence determinations (15).

Trypsin (type III-S, bovine pancreas), nucleotides, and ouabain were obtained from Sigma. CPM was purchased from Molecular Probes. ⁸⁶Rb⁺ and ²²Na⁺ were obtained from DuPont NEN.

RESULTS

Partial Inactivation of the Rb⁺ Occlusion Capacity of the Digested Enzyme—Or *et al.* (6) reported that the trypsin-digested enzyme lost more than 80–90% of its capacity to occlude Rb⁺ when it was incubated at 37 °C for 10 min in the absence of Rb⁺. Lutsenko *et al.* (12) confirmed the functional instability of the digested preparation under these conditions; however, their data showed that only half of the Rb⁺ occlusion capacity was subject to time-dependent inactivation at 37 °C. Our experiments (Fig. 1) also showed that loss of Rb⁺ occlusion capacity plateaued at about 50% of the control value. Lutsenko *et al.* (12) did not study the properties of the remaining half of occluded Rb⁺.

Affinities of Rb⁺ Sites of the Digested Enzyme Before and After Partial Inactivation—Our previous studies showed that to determine the affinity of the native enzyme for Rb⁺ at 4 °C, it was necessary to incubate the enzyme with different Rb⁺ concentrations for about 24 h to reach maximal equilibrium binding levels (5). Such long incubation periods were also necessary for the two digested preparations, as exemplified by the data of Fig. 2a showing the time courses of binding to the two preparations at 500 μ M Rb⁺. In experiments of Fig. 2b we used the two digested preparations and determined bound Rb⁺ after 24 h of incubation at 4 °C at varying Rb⁺ concentrations. The

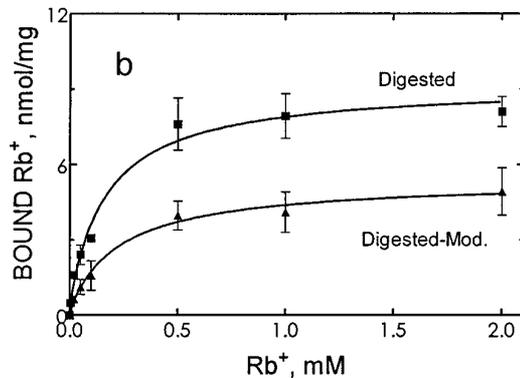
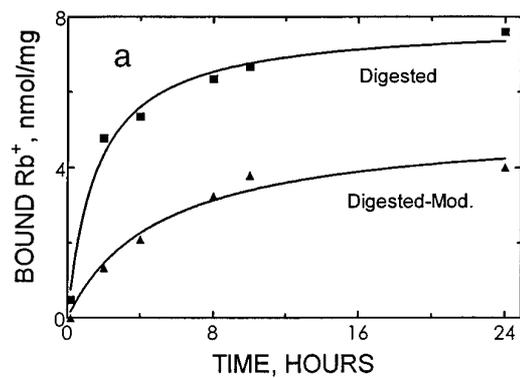


FIG. 2. *a*, time course of the occlusion of Rb⁺ by the digested enzyme and the digested modified enzyme. The preparations were incubated at 4 °C with 500 μ M ⁸⁶Rb⁺ and assayed at the indicated times. *b*, effects of varying Rb⁺ concentrations on Rb⁺ occlusion by the digested and the digested modified enzymes. The preparations were incubated at 4 °C with the indicated ⁸⁶Rb⁺ concentrations and assayed after 24 h. The indicated values are the means \pm S.E. of four separate experiments.

results showed that $K_{0.5}$ of Rb⁺ was nearly the same (about 50 μ M) in both preparations but that the maximal binding level of the digested modified preparation was about half of the level of the original digested preparation (Fig. 2b). Clearly, the remaining Rb⁺ binding after partial inactivation represents highly specific occlusion at sites having similar affinities to those of the occlusion sites of the original digested enzyme.

ATP Effects on Rb⁺ Deocclusion and Occlusion Kinetics—In the native enzyme, ATP at a low affinity site increases the rates of deocclusion and occlusion of Rb⁺ (5). The following experiments were done to see if such effects of ATP persist in the digested preparations.

Comparison of the spontaneous Rb⁺ deocclusion rates of the native enzyme and the two digested enzyme preparations at 4 °C are shown in Fig. 3a. Deocclusion rate of the digested enzyme was greater than that of the native enzyme. After half of the occlusion capacity of the digested enzyme was inactivated, the deocclusion rate of the remaining half was reduced to a level below that of the native enzyme (Fig. 3a). ATP (2 mM) stimulated deocclusion in both digested preparations (Fig. 3b), but the ATP effect was clearly more dramatic on the digested modified preparation.

In experiments of Fig. 4, the stimulatory effects of varying ATP concentrations on Rb⁺ release from the digested modified preparation were examined. $K_{0.5}$ of ATP was 300 μ M when Rb⁺ was released into a Rb⁺-free medium (Fig. 4). The presence of 20 mM unlabeled Rb⁺ in the medium had no effect on the spontaneous rate of release of occluded Rb⁺ (data not shown), but the $K_{0.5}$ of ATP for stimulation of Rb⁺ release was reduced to 25 μ M when deocclusion occurred into a medium containing

¹ The abbreviations used are: CPM, 7-diethylamino-3-(4-maleimidyl-phenyl)-4-methylcoumarin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

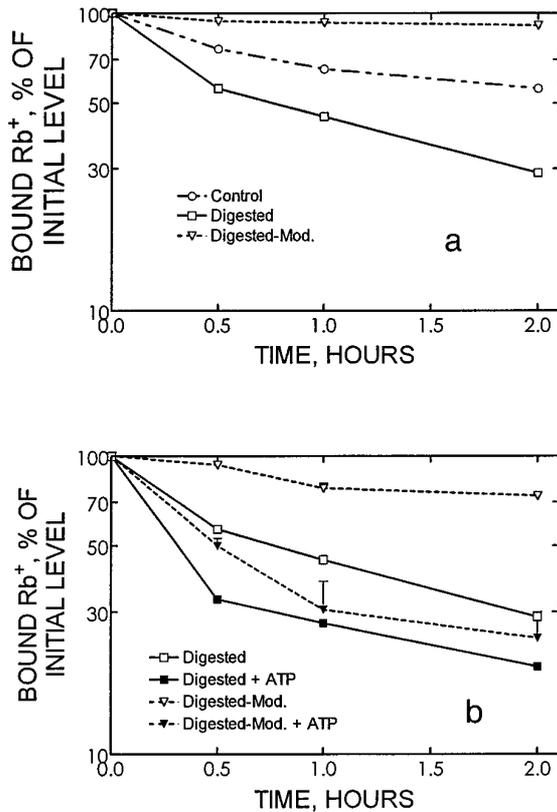


FIG. 3. *a*, spontaneous deocclusion rates of occluded Rb⁺ from the control enzyme, the digested enzyme, and the digested modified enzyme. The preparations were incubated with 5 μM ⁸⁶Rb⁺ at 4 °C for 24 h. After dilution, deocclusion rates were determined as indicated under "Experimental Procedures." *b*, effects of ATP on the release of occluded Rb⁺ from the digested and the digested modified enzymes. The preparations were incubated with 5 μM ⁸⁶Rb⁺ at 4 °C for 24 h; deocclusion rates were determined in the absence and the presence of 2 mM ATP. The indicated values are the means ± S.E. of six different experiments. When S.E. is not shown, it was smaller than the size of the symbol.

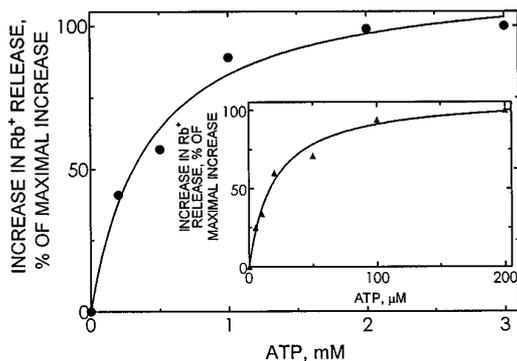


FIG. 4. Effects of different concentrations of ATP on the release of occluded Rb⁺ from the digested modified enzyme. ⁸⁶Rb⁺ was occluded as indicated in Fig. 3. ⁸⁶Rb⁺ release into a Rb⁺-free medium was measured after 1 h in the absence of ATP and in the presence of indicated ATP concentrations. The *inset* shows the results of a similar experiment when ⁸⁶Rb⁺ was released into a medium containing 20 mM unlabeled Rb⁺.

20 mM Rb⁺ (Inset to Fig. 4). This effect of medium Rb⁺ on the apparent affinity of ATP is similar to that noted before in the native enzyme (16).

When experiments similar to those of Fig. 4 were done on the effects of ATP on Rb⁺ release from the unmodified digested enzyme, the results (not shown) were nearly superimposable on those of Fig. 4. In experiments similar to those of Figs. 3*b* and

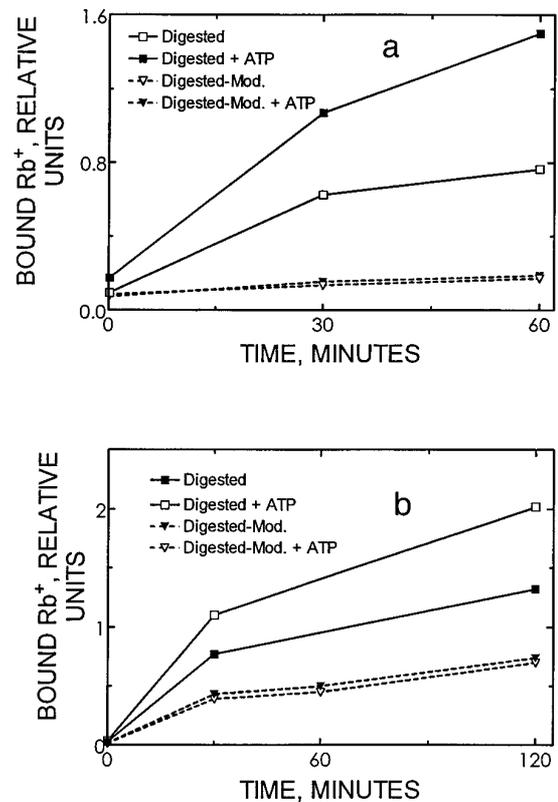


FIG. 5. Effects of ATP on the time course of Rb⁺ occlusion by the digested and the digested modified enzymes. The preparations were incubated at 4 °C with 5 (*a*) or 200 μM ⁸⁶Rb⁺ (*b*) in the absence or the presence of 2 mM ATP, and occluded Rb⁺ was assayed at indicated times.

4, we compared the effects of ATP with those of other nucleotides on Rb⁺ release from both digested preparations. The data (not shown) indicated that ADP and CTP, but not AMP and GTP, stimulated Rb⁺ deocclusion. Thus, the specificity of the low affinity nucleotide effect on the two digested preparations is the same as that of the native enzyme (5).

In experiments of Fig. 5 effects of ATP on the initial rates of Rb⁺ occlusion at 4 °C in the two digested preparations were examined. At both Rb⁺ concentrations used (5 and 200 μM) the initial rates in the absence of ATP were higher in the unmodified digested enzyme (Fig. 5). At both Rb⁺ concentrations, ATP stimulated the initial rates in the unmodified digested enzyme but not in the digested modified preparation (Fig. 5). Using the unmodified digested enzyme, we established (data not shown) that ATP stimulation of Rb⁺ occlusion was also a low affinity effect of ATP and had the same nucleotide specificity as the ATP stimulation of Rb⁺ occlusion rate in the native enzyme (5).

Ouabain Effects on Rb⁺ Occlusion-Deocclusion in the Digested Enzymes—As in the native enzyme (5), ouabain decreased Rb⁺ occlusion and deocclusion rates in both digested preparations, as exemplified by the data of Fig. 6. These ouabain effects were saturable (Fig. 6, *inset*), with apparent ouabain affinities significantly lower than the binding constant of ouabain for the native canine enzyme as determined by equilibrium binding studies under optimal conditions (13). As in the native enzyme (5), ouabain blocked the stimulatory effect of ATP on the Rb⁺ occlusion rate in the digested enzyme (data not shown).

Na⁺ Effects on Rb⁺ Occlusion-Deocclusion in the Digested Enzymes—In the native enzyme, Rb⁺ occlusion is competitively inhibited by Na⁺ (5, 17). However, Na⁺ also binds to the native enzyme containing occluded Rb⁺ and accelerates the rate of

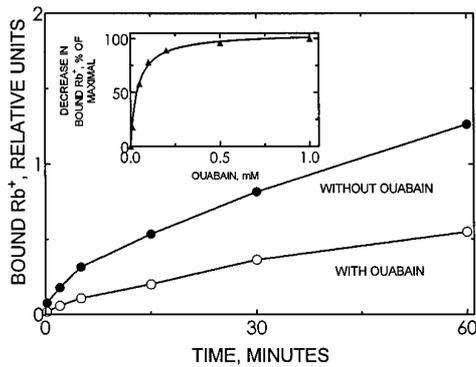


FIG. 6. Effect of ouabain on Rb⁺ occlusion rate by the digested enzyme. The digested enzyme was preincubated for 20 min at 24 °C in the absence of ouabain or in the presence of 1 mM ouabain. The preparations were then incubated at 4 °C with 5 μ M ⁸⁶Rb⁺ and assayed for occluded Rb⁺. Inset, the results of similar experiments in which ouabain concentration was varied, and occlusion was measured after 60 min.

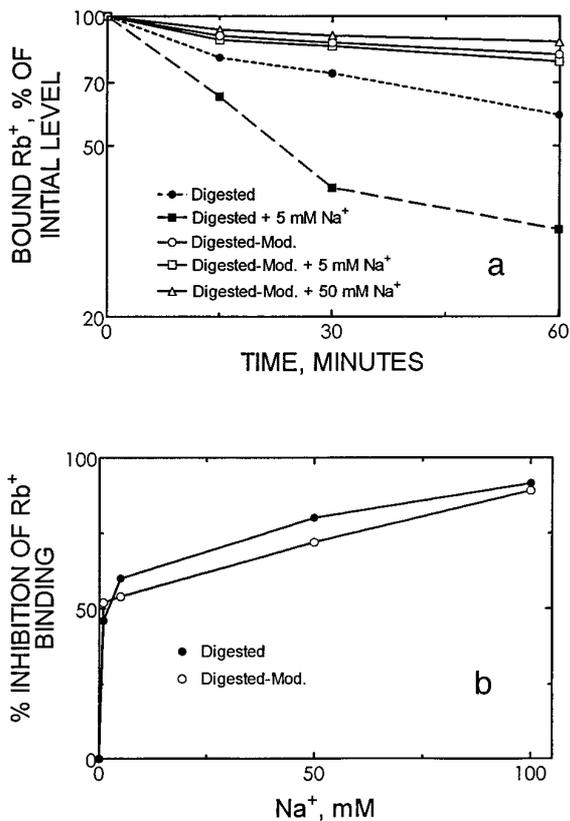


FIG. 7. *a*, effects of Na⁺ on the time courses of Rb⁺ release from the digested and the digested modified enzymes. ⁸⁶Rb⁺ was occluded as indicated in Fig. 3, and deocclusion rates were determined by incubation at 4 °C in medium free of Na⁺ and containing the indicated Na⁺ concentrations. *b*, inhibition of Rb⁺ occlusion by Na⁺ in the digested and the digested modified enzymes. The preparations were incubated with 200 μ M ⁸⁶Rb⁺ at 4 °C in medium containing no Na⁺ or the indicated Na⁺ concentrations. Rb⁺ occlusion was assayed after 24 h.

Rb⁺ deocclusion (5). The latter allosteric effect of Na⁺ on Rb⁺ deocclusion was clearly observed on the digested preparation but not in the digested modified preparation (Fig. 7*a*). In both digested preparations, however, Rb⁺ occlusion was similarly inhibited by Na⁺ (Fig. 7*b*).

Na⁺ Occlusion Capacities of the Digested Enzyme—A portion of a control native enzyme preparation was trypsin-digested, and a portion of this digested preparation was converted to the digested modified preparation as described under “Experimental Procedures.” Na⁺ occlusion capacities of the three related

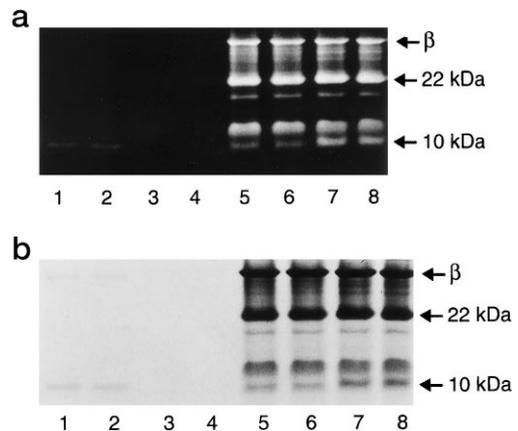


FIG. 8. Structural changes induced by the partial inactivation of the occlusion capacity of the digested enzyme. A sample of the digested enzyme was partially inactivated as in Fig. 1, and an equal amount of the digested enzyme (control) was similarly treated in the presence of Rb⁺ to prevent inactivation. After centrifugation, each supernatant or washed pellet was labeled with fluorescent CPM as indicated under “Experimental Procedures,” and comparable portions were resolved on 16.5% Tricine gels in duplicate (*a*). Comparable portions of the supernatants and the pellets were similarly treated without exposure to CPM, resolved on the same gels in duplicate, and stained with Coomassie Blue (*b*). Lanes 1 and 2, supernatant of the partially inactivated sample. Lanes 3 and 4, control supernatant. Lanes 5 and 6, partially inactivated pellet. Lanes 7 and 8, control pellet.

preparations were then compared in the presence of oligomycin, using 10 mM ²²Na⁺, which is known to be near-saturating for Na⁺ occlusion by the native and the digested enzymes (8). In three determinations the values (nmol/mg \pm S.E.) were: native enzyme, 6.2 \pm 0.3; digested enzyme, 13.7 \pm 0.5; and digested modified enzyme, 4.5 \pm 0.3. Evidently, the same treatment that causes inactivation of half of the Rb⁺ occlusion capacity of the digested enzyme (Figs. 1 and 2*b*) also causes the loss of two-thirds of its Na⁺ occlusion capacity. That the Na⁺ occlusion capacity of the digested enzyme is about double that of the native enzyme is in keeping with the loss of about half of the protein content of the native enzyme due to trypsin digestion (8).

Peptide Compositions of the Digested Enzymes—After the digested enzyme was treated under partial inactivating conditions and centrifuged (Fig. 1), the supernatant was saved and concentrated, and the pellet was washed as described under “Experimental Procedures.” A control sample of the digested enzyme that was incubated in the presence of Rb⁺ and was not inactivated (Fig. 1) was similarly treated. The supernatants and the pellets were then labeled with CPM and resolved on Tricine/SDS gels (Fig. 8*a*). Alternatively, the samples that were treated similarly but not exposed to CPM were resolved on the same gels and stained with Coomassie Blue (Fig. 8*b*).

As evident from the gel patterns of the pellets (Fig. 8, lanes 5–8), the most obvious difference between the two preparations is the lower intensity of a band with the apparent molecular mass of 10-kDa in the digested modified preparation. When all other stained bands of the pellets (Fig. 8*b*, lanes 5–8) were quantitated by densitometry, no significant differences between the digested and the digested modified samples were noted (data not shown). It is also evident from Fig. 8 (lanes 1–4) that the supernatant of the digested modified preparation, but not that of the control, contains a 10-kDa band. These results are in general agreement with those of Lutsenko *et al.* (12).

When the 10-kDa band of the supernatant (combined from several gels such as those of Fig. 8*b*, lanes 1 and 2) was subjected to N-terminal analysis, one prominent sequence of QAADMILLDDN was found confirming the findings of

TABLE I

Loss of two α -fragments containing H₃-H₄ and H₅-H₆ transmembrane domains from the digested enzyme whose occlusion capacity is partially inactivated.

N-terminal sequences of the bands designated as 10-kDa in the control digested enzyme (Fig. 8*b*, lanes 7 and 8) and in the partially inactivated digested enzyme (Fig. 8*b*, lanes 5 and 6) were determined as indicated under "Experimental Procedures." The yields of the three prominent sequences found were estimated from the average elevation of the residues in cycles 2–11.

Peptide	Sequence	Yield	
		Before partial inactivation	After partial inactivation
		<i>pmol</i>	
H ₃ -H ₄	IATLASGLEGG	106	35
H ₅ -H ₆	QAADMILLDDN	71	30
γ -Subunit	GDVDPFYDYE	55	54

Lutsenko *et al.* (12) and indicating that partial inactivation of occlusion capacity is accompanied by the release of the α -fragment containing the H₅-H₆ transmembrane domains. To determine if the release of this fragment accounted for the different intensities of the 10-kDa bands of the pellets of the digested and the digested modified samples (Fig. 8*b*, lanes 5–8), these bands obtained from equivalent amounts of the two samples were also subjected to N-terminal analysis. The findings (Table I) showed that the band from each sample contained three prominent N termini corresponding to those of the putative γ -subunit (9, 14), the α -subunit fragment containing the H₃-H₄ transmembrane domain (9), and the α -subunit fragment containing the H₅-H₆ transmembrane domain (9, 12). Comparison of the yields (Table I) indicated that partial inactivation caused no change in the content of γ but that it led to 60–70% reductions in the contents of both α -subunit fragments within the digested membrane preparation.

DISCUSSION

Two Dissimilar Cation Occlusion Pockets—It is well established that during a cycle of ATP hydrolysis, Na⁺/K⁺-ATPase catalyzes the antiports of 3Na⁺ and 2K⁺ across the cell membrane (1), and most evidence indicates that isolated preparations of the enzyme occlude 3Na⁺, 2K⁺, 2Rb⁺, or 2Tl⁺ per active site (3–5, 18). It is also known that the trypsin-digested enzyme we have used in these studies retains the full capacity of the intact enzyme for occlusions of 3Na⁺ or 2Rb⁺ (8). The data presented here show that under specified conditions where half of the Rb⁺ occluding capacity of the digested enzyme is readily inactivated, the other half is resistant to inactivation (Figs. 1 and 2), clearly indicating that the preparation contains two dissimilar pools of occluded Rb⁺. The dissimilarity of the two Rb⁺ pools is also indicated by the different spontaneous occlusion-deocclusion kinetics of the two (Figs. 3 and 5). In the past, the two Rb⁺ sites of the enzyme have been assumed to be within the same occlusion pocket (4, 6). Although the different kinetics of the two Rb⁺ pools may be accommodated by two sites within the same pocket (4), it is difficult to imagine different sensitivities of two adjacent sites within the same pocket to the inactivating conditions used. That the two Rb⁺ pools are not within the same occlusion pocket is also supported by the findings that the occlusion-deocclusion rates of the two pools are regulated differently by ATP and Na⁺ (see below). Although our data on Na⁺ occlusion are more limited, they also suggest dissimilar pools of occluded Na⁺ within the digested enzyme by showing that inactivation of the half of Rb⁺ occlusion capacity is accompanied by the inactivation of two-thirds of Na⁺ occlusion capacity ("Results"). Based on the above considerations we conclude that there are two distinct cation occlusion pockets within the trypsin-di-

gested preparation: the labile pocket containing one Rb⁺ and two Na⁺ sites and a stable pocket containing one Rb⁺ site and one Na⁺ site. That Na⁺ and Rb⁺ compete for the binding sites within each pocket is suggested by similar inhibitory effects of Na⁺ on Rb⁺ occlusion by the digested enzyme before and after partial inactivation (Fig. 7*b*). Both pockets are similarly sensitive to ouabain (Fig. 6 and "Results").

Regulation of the Access Channels of the Two Rb⁺ Occlusion Pockets by ATP and Na⁺—Our data showing that the digested enzyme exhibits a low affinity ATP site whose occupation affects Rb⁺ occlusion-deocclusion kinetics seem to resolve one of the long-standing controversies on the relationship between the high and low affinity ATP sites of the enzyme (19, 20) in favor of the view that the two are distinct entities. Of the nine residues of the α -subunit that have been implicated in high affinity ATP binding by chemical modification and site-directed mutagenesis (21, 22), only one (Lys⁷⁶⁶) remains in the digested enzyme. Clearly, it is no longer reasonable to assume that the two sites are different conformational states of the same binding domain. It should now be possible to use the digested preparations lacking the high affinity catalytic site for the characterization of the residues involved in the allosteric ATP binding site.

In the digested enzyme prior to partial inactivation, ATP increases both occlusion and deocclusion rates of Rb⁺ (Figs. 3 and 5) as it does in the native enzyme (5). Previously, we interpreted this dual effect of ATP in the native enzyme as ATP-induced widening of an access channel through which Rb⁺ had to pass either to reach the Rb⁺ binding sites deep within the pocket or to reach the medium when released from the binding site (5). An intriguing modification of this is suggested by the finding that after partial inactivation of the digested enzyme, ATP increases Rb⁺ deocclusion rate (Fig. 3) but not the initial rate of Rb⁺ occlusion (Fig. 5). Evidently, in the stable pocket, an exit channel is opened by ATP only when the sites are occupied by Rb⁺. If this is the case, to explain the dual effects of ATP on occlusion-deocclusion rates of the combination of the two pockets, it is sufficient to assume that in the labile pocket ATP opens only an entry channel into the empty pocket. Thus, ATP seems to couple the filling of one pocket to the emptying of the other. This may explain the synergistic effect of medium Rb⁺ (or K⁺) on ATP stimulation of Rb⁺ deocclusion (which appears as a decrease in apparent K_m of ATP at the low affinity site) in the digested enzyme (Fig. 4) and in the native enzyme (16).

Na⁺ increases the deocclusion rates of Rb⁺ in the unmodified digested enzyme (Fig. 7*a*) and in the native enzyme (5). In the stable Rb⁺ occlusion pocket, however, Na⁺ has no effect on the deocclusion rate (Fig. 7*a*). This suggests that the allosteric Na⁺ effect on Rb⁺ deocclusion may be limited to the labile pocket. A more appealing alternative explanation is that Na⁺ in one pocket accelerates deocclusion of Rb⁺ from the other. Thus, the lack of Na⁺ effect on Rb⁺ deocclusion from the stable pocket in experiments of Fig. 7*a* would be due to the absence of the labile pocket. This alternative is supported by the data showing that $K_{0.5}$ of Na⁺ for its allosteric effect on Rb⁺ deocclusion (5) is about the same as $K_{0.5}$ values for Na⁺ occlusion (8) and Na⁺ inhibition of Rb⁺ occlusion (Ref. 5 and Fig. 7*b*).

Structural Considerations Related to the Two Occlusion Pockets—The occlusion capacity of the digested enzyme used here is due to a peptide complex consisting of four fragments of α -subunit containing all its ten transmembrane helices, an essentially intact β -subunit, and perhaps the γ -subunit (9, 10, 14). The schematic presentation of the digested preparation without the γ -subunit is shown in Fig. 9. Lutsenko *et al.* (12) reported that inactivation of half of the occlusion capacity of

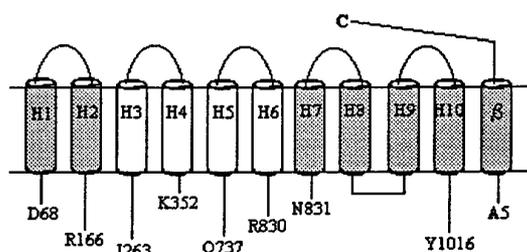


FIG. 9. Suggested locations of the two Rb⁺ occlusion pockets within the peptide complex of the trypsin-digested enzyme. The indicated N termini have been determined experimentally, but most of the indicated C termini have been estimated based on apparent molecular masses (9, 10, 15, 29). The labile pocket is associated with a complex of H₃-H₄ and H₅-H₆ helices. The stable pocket is located within the complex of H₁-H₂, H₇-H₁₀, and β helices.

this preparation was accompanied by the release of the α -fragment containing the H₅-H₆ hairpin (Fig. 9) from the peptide complex and that this release seemed to be complete. Although our data confirm the solubilization of this fragment (Fig. 8 and Table I), they indicate that the α -fragment containing the H₃-H₄ helices (Fig. 9) is also lost from the partially inactivated membranes and that the loss of neither fragment is complete; *i.e.* the digested modified preparation retains about 30–40% of each fragment (Table I). A reasonable explanation of our findings is that the partial inactivation of the occluding capacity of the digested enzyme is accompanied by the simultaneous release of these two fragments and that our inability to detect the H₃-H₄ fragment in the supernatant may be due to the fact that whereas the released and aggregated H₅-H₆ fragment is water soluble (12), the released and aggregated H₃-H₄ is not and is easily lost during the procedures used for the isolation and detection of the released peptides. This working hypothesis needs to be tested by further experiments.

How do the above structural changes lead to inactivation of one occlusion pocket and retention of the other? There are two alternative explanations. First, because the occluding capacity of the digested enzyme is due to a complex of interacting peptides, one may assume that there are two such complexes: one that loses the two peptides containing H₃-H₆ domains upon incubation at 37 °C and the other that retains these peptides. If this is the case, the labile and the stable pockets, with their different properties, must be on two dissimilar complexes that, by inference, must be the products of two dissimilar α -subunits. This is not a likely possibility. Although the existence of two functionally dissimilar α -subunits in the native enzyme seems to be established by our recent studies (23), this asymmetry is most likely due to α - α interactions (19, 20, 24). However, structural regions of the α -subunit on the large intracellular central loop that are presently known to be involved in α - α contact (24–26) are no longer present in the digested enzyme, and electron microscopic studies on the digested enzyme also suggest the absence of α - α associations in this preparation (27). The more likely explanation is that the structural elements of the two distinct occlusion pockets are within a single copy of the large peptide complex, the two α -fragments containing the H₃-H₆ domains being associated with the labile pocket (Fig. 9) and the remainder of the large complex (the 22-kDa C-terminal fragment of α , the 11-kDa N-terminal fragment of α , and the β -subunit) being associated with the stable pocket (Fig. 9). To account for the fraction of H₃-H₆ domains that remains with the membrane after partial inactivation, we may assume that the critical step in the inactivation of the labile pocket is the disruption of interaction between the H₃-H₆ domains and the rest of the complex but not the complete exit of these fragments from the bilayer.

The following points regarding the proposed locations of the

two occlusion pockets are worthy of further note. First, there is ample evidence based on chemical modification and site-directed mutagenesis studies to indicate the involvement of H₃-H₄ and H₅-H₆ domains in cation binding and transport (12, 21, 22, 28).

Second, although in the above analysis we arrived at the location of the stable pocket by the process of elimination, recent studies on the three-dimensional packing of the transmembrane helices of the enzyme do indicate close contact between the H₁-H₂ and H₇-H₁₀ helices of α and the single transmembrane helix of β (15, 29). Also, chemical modification experiments and some site-directed mutagenesis studies suggest the involvements of the H₈ and H₉ transmembrane domains in cation transport by Na⁺/K⁺-ATPase and the related gastric H₄/K⁺-ATPase (30, 31), and a role of β in the regulation of K⁺ binding is well established (10, 32, 33). Concerning the role of β in one of the two proposed occlusion pockets, it is also of interest that studies of Lutsenko and Kaplan (10) showed that dithiothreitol-induced reductions of the disulfides of the extracellular domain of β were accompanied by the loss of half of the Rb⁺ occlusion capacity of the enzyme. Because our recent work (29) indicates that structural changes in the extracellular domain of β are transmitted to its single transmembrane helix and through this to the interacting H₁-H₂ and H₇-H₁₀ transmembrane helices of α , it is reasonable to suspect that treatment with dithiothreitol may cause the selective inactivation of the occlusion pocket that we have designated as the stable pocket based on the experiments presented here. Studies to test this possibility are in progress.

Third, the similar ouabain sensitivities of the two occlusion pockets noted here are consistent with the involvements of multiple transmembrane and extracellular domains of α -subunit in the regulation of ouabain interaction with the enzyme (34).

Implications for the Reaction Mechanism of the Na⁺/K⁺-ATPase—As discussed before (5), the Albers-Post scheme for the transports of Na⁺ and K⁺ coupled to ATP hydrolysis by this enzyme considers neither the heterogeneity of the access channels of the occlusion pockets nor all of the allosteric ligand effects on these access channels. We may now add that this commonly used reaction mechanism also does not include the consideration of two separate but interacting K⁺ (Rb⁺) occlusion pockets with distinctly different ATP-sensitive access channels. Should the existence of such multiple cation occlusion pockets be verified, we suggest that further characterizations of their comparative properties, not only in the inactive fragments of the enzyme but also in the active native enzyme, will be necessary before their inclusion in the transport cycle of the enzyme is attempted.

Acknowledgments—We thank Dr. J. Pohl, Emory University Microsequencing Facility, for the N-terminal analyses.

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