An Essential Role for the Extracellular Domain of the Na,K-ATPase β-Subunit in Cation Occlusion†

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ABSTRACT: The role of the Na,K-ATPase β-subunit in stabilization of ion-binding sites has been investigated. Treatment of the purified renal Na,K-ATPase with 0.25 M DTT at 40 °C for 1 h resulted in 50% loss of Rb occlusion, which correlates with partial reduction of S–S bridges in the extracellular portion of the β-subunit; both of these effects were prevented by the presence of 20 mM RbCl. To clarify the role of the extracellular portion of the β-subunit, "19-kDa membranes" (Na,K-ATPase posttryptic residues, which have been shown to possess many of the cation-binding properties) were used. Incubation of the "19-kDa membranes" with 0.2 M DTT for 1 h at 37 °C abolished 70–80% of the ^{86}Rb occlusion capacity. This was accompanied by accumulation of 16- and 17-kDa peptides (in SDS–PAGE of the membranes) and release of a 45-kDa band derived from the Na,K-ATPase β-subunit to the supernatant. The appearance of the 45-kDa fragment of the β-subunit in the supernatant confirms the existence of only one transmembrane fragment in this subunit. N-Terminal sequence analysis of the 16- and 17-kDa bands revealed the same structure, A-K-E-E-G-, which corresponds to the β-subunit sequence beginning at Ala^5. The simultaneous presence of 25 mM RbCl (but not 25 mM choline chloride) during DTT treatment prevents almost all (85%) of the loss of Rb occlusion, the appearance of 16- and 17-kDa bands, and reduction and release of the 45-kDa fragment. Fluorescent labeling with CPM [3-(4-maleimidophenyl)-4-methyl-7-(diethylamino)coumarin] of "19-kDa membranes" following DTT treatment revealed no changes either in the "19-kDa peptide" or in any peptides smaller than 11 kDa. Similar procedures were used on Pronase-digested "19-kDa membranes". DTT treatment and CPM reaction following Pronase digestion revealed changes in both the mobility and fluorescence intensity of a 45-kDa peptide derived from the β-subunit concomitant with loss in the Rb occluding capacity. We conclude that the β-subunit of the Na,K-ATPase participates in stabilizing the occluded cation intermediate and that the extracellular domain structure of the β-subunit which is maintained by S–S bridges plays an important role in the K-binding function.

The cellular composition of Na and K ions is maintained in most eukaryotic cells by the operation of the Na pump or Na,K-ATPase (EC 3.6.3.37) (Glynn, 1985; Kaplan, 1989). The Na,K-ATPase is an oligomeric intramembrane protein consisting of at least two subunits. The catalytic α-subunit (112 kDa) has been shown to be phosphorylated during the pumping cycle and is the polypeptide for which most evidence has been accumulated for the locus of binding of physiological ligands (Pedemonte & Kaplan, 1990; Kaplan, 1992). The significance of the β-subunit (a heavily glycoslyated protein of M_r 55K) in the normal functioning of the Na pump remains an unanswered question. A variety of studies on the Na,K-ATPase have suggested the importance of interactions between the α- and β-subunits in order to provide a membrane-inserted, fully active complex (McDonough et al., 1990).

Reduction of S–S bridges (Kawamura & Nagano, 1984) or mutations of cysteine residues have been shown to result in loss of ATPase activity (Kawamura & Noguchi, 1992). Extracellular localization of the S–S bridge-containing portion of the β-subunit excludes its direct participation in the formation of the ATP-binding domain. Furthermore, ATP (even at 5 mM) does not protect against DTT-affected inactivation. On the other hand, monovalent cations specifically (K ≫ Na) protect the enzyme against DTT-induced inactivation and β-subunit reduction (Kawamura & Nagano, 1984; Kirley, 1990). It has been suggested that interaction/stabilization of ion-binding sites might be affected by the conformation of the β-subunit (Kirley, 1990) but no direct evidence was available. The opposite conclusion about participation of the β-subunit in ion binding was made recently, based on the results of experiments on exhaustive proteolytic digestion (Capasso et al., 1992). According to electrophoretic data, the extracellular portion of the β-subunit was removed by Pronase treatment without any changes in the Rb occlusion capacity; thus, all ion-binding properties were assigned to the α-subunit intramembrane segments. We attempted to resolve this controversy by measuring Rb occlusion after DTT treatment for whole enzyme and for postproteolytic fragments. If the extracellular portion of the β-subunit was not important for ion occlusion, the reduction of S–S bridges in this domain should have no effect on the enzyme–Rb-binding capacity. However, this was not the case. In the present work, we provide evidence that structural disruption of the extracellular portion of the β-subunit drastically affects ion binding and we suggest that the β-subunit plays an important role in the K-binding process. Parts of this work have been presented previously (Lutsenko & Kaplan, 1992).

EXPERIMENTAL PROCEDURES

Dithiothreitol, DPCC-treated trypsin, soybean trypsin inhibitor, adenosine 5′-triphosphate (Tris salt and disodium salt), Pronase, sucrose, ultrapure urea, Trizma base, Tricine, and Dowex-50W were obtained through Sigma. β-Mercaptoethanol, sodium dodecyl sulfate, ammonium persulfate, Coomassie R-250, and low molecular weight standards were from Bio-Rad. Acrylamide and bis(acrylamide) were obtained through Boehringer. Centricon 30 and Centricon 10 microconcentrators were from Amicon. ^{86}Rb and rainbow gel

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electrophoresis standards (2.5–46 kDa) were purchased from Amersham. 3-(4-Maleimidophenyl)-4-methyl-7-(diethylamino)coumarin (CPM) was from Molecular Probes, Inc. Poly(vinylidene difluoride) (PVDF) membrane was from Millipore.

General Procedures. Na,K-ATPase was purified from dog kidney according to Jorgensen (1975) with an average specific activity of 24–26 μmol of P_i (mg of protein)$^{-1}$ min^{-1} assayed according to Brotherus et al. (1981). Membrane protein concentrations were determined using the method of Lowry (Lowry et al., 1951) with bovine serum albumin as standard. Rb occlusion was measured essentially as described previously (Shani et al., 1987). The standard Rb occlusion assay medium (40 μL) contained 30 μg of Na,K-ATPase or "19-kDa membrane" protein and 0.01–5 mM RbCl plus (1.5–2.0) × 10^6 cpm of ^{86}Rb in 25 mM imidazole hydrochloride/1 mM EDTA, pH 7.5, or in 100 mM Tris-HCl, pH 6.95. In experiments with "19-kDa membranes", buffers always contained 2 mM RbCl. After 10 min at 37 °C, 500 μL of 250 mM ice-cold sucrose solution was added, the suspension was transferred to Dowex-50W columns, and enzyme with occluded ^{86}Rb was eluted with 1 mL of 250 mM sucrose solution.

The Na,K-ATPase or "19-kDa membranes" (see below) were separated in 10% or 15% polyacrylamide gels, containing 0.1% SDS and 2 M urea, essentially as in Laemmli (1970). Control and DTT-treated "19-kDa membranes" were also separated in Tricine gels according to Schagger and Von Jagow (1987). Transfer of protein bands onto PVDF membrane was performed as reported (Matsudaira, 1987), by electroblotting in 10 mM CAPS/10% methanol, pH 11. After transfer, the membrane was stained with 0.1% Coomassie R-250 in 50% methanol for 2 min, destained by washing several times in 50% methanol/10% acetic acid solution, and rinsed with water, and N-terminal amino acid sequencing was performed.

Treatment with DTT. Na,K-ATPase (1 mg/mL) was incubated in 25 mM imidazole/1 mM EDTA, pH 7.5 (buffer A), with 0.25 M DTT at 40 °C for various time periods. In some experiments, 5 mM ATP, Tris salt, or 20 mM RbCl was included in the incubation media. Aliquots of 65 μL were taken at various times, diluted with 335 μL of ice-cold buffer A, and sedimented at 353000*g*, 4 °C, for 10 min (Beckman TL-100 ultracentrifuge). The pellets were resuspended in 100 μL of ice-cold buffer A, then diluted with 900 μL of the same buffer, and sedimented at 353000*g*, 4 °C, for 30 min. The pellets were resuspended in 65 μL of 0.1 M Tris-HCl buffer, pH 6.95, 2 × 30 μL samples were used to measure ^{86}Rb occlusion, and 5 μL was taken to measure ATPase activity. Treatment of "19-kDa membranes" with DTT and washing procedures were performed under the same conditions as native enzyme but in the presence of 2 mM RbCl.

Preparation of "19-kDa Membranes". "19-kDa membranes" were prepared by trypsin treatment of intact enzyme (Karlish et al., 1990). Na,K-ATPase at 1.5 mg/mL was suspended in media containing 25 mM histidine, 1 mM EDTA–Tris, and 10 mM RbCl, pH 7.5, and incubated at 37 °C for 1 h with DPCC–trypsin (1:10 w/w with respect to Na,K-ATPase). Soybean trypsin inhibitor was added (5:1 w/w with respect to trypsin) and incubated with the protein for 10 min at 37 °C. The suspension was diluted to 24 mL with buffer containing 25 mM imidazole, pH 7.5, 1 mM EDTA–Tris, and 2 mM RbCl, and membranes were collected by centrifugation at 250000*g* for 1 h (Beckman TI-60 rotor) at 4 °C. The membranes were homogenized in the latter buffer, and centrifugation was repeated twice. Digestion with Pronase and proteinase K followed the procedures of Capasso et al. (1992). Control enzyme or "19-kDa membranes" were suspended in buffer containing 25 mM imidazole hydrochloride, pH 7.5, 10 mM EDTA–Tris, and 5 mM RbCl and incubated at 37 °C for 1 h with Pronase (1:10 or 1:3 w/w, respectively), or native enzyme was incubated at 37 °C for 1 h with proteinase K (1:20 w/w). In each case, the suspension was diluted with ice-cold 25 mM imidazole, 1 mM EDTA, and 5 mM RbCl, pH 7.5, buffer containing 1 mM phenylmethanesulfonyl fluoride and centrifuged as described above, and the dilution and washing procedures were repeated twice.

Labeling with CPM. After DTT treatment, Na,K-ATPase or "19-kDa membranes" were washed twice with 25 mM imidazole hydrochloride, 1 mM EDTA, and 2 mM RbCl buffer, pH 7.5, then mixed with an equal volume of 10% SDS, vortexed, and incubated at room temperature for 10 min; 5–10 μL of an acetone solution of CPM (10 mg/mL) was added to label 30–40 μg of enzyme or 100–120 μg of "19-kDa membrane". The reaction mixture was vortexed and incubated at room temperature in the dark for 1 h. The samples were then mixed with 10 volumes of ice-cold methanol and incubated at −20 °C for at least 2 h. The protein was pelleted by centrifugation at 475000*g*, 10 min, 2 °C, or at 5000*g*, 60 min, 2 °C. Pellets were redissolved in 8 M urea/10% SDS/125 mM Tris buffer, pH 6.8 (1:1:1 v/v), and applied to a 15% Laemmli gel, containing 2 M urea, or a Tricine gel. When sequencing of the protein band was subsequently performed, urea was omitted, and the separating gel was preelectrophoresed for 5 h with separating buffer, pH 8.8, with 20 mM β-mercaptopropionic acid. After electrophoresis, protein fragments were transferred onto a PVDF membrane as described above. For CPM labeling of the fragments, which were washed away during reduction and sedimentation, the supernatants after the first centrifugation were mixed with ice-cold methanol (1:5 v/v) to remove most of the DTT. After overnight incubation at −20 °C, aggregated fragments were collected by centrifugation at 47500*g*, 15 min, 2 °C. Pellets were resuspended in 8 M urea/10% SDS/imidazole–EDTA buffer, pH 7.5 (1:1:1 v/v), and were labeled with CPM as described above.

In the determination of the extent of reduction of the β-subunit, the Na,K-ATPase samples after reduction (in the presence of 10 mM ATP) were washed 3 times by centrifugation and resuspension in 25 mM imidazole/1 mM EDTA, pH 7.5 (see above), and resuspended in the same buffer, an equal volume of 10% SDS was added, and the protein was then modified with CPM, as described above. After 1 h, urea (8 M) and Laemmli sample buffer were added to the reaction mixture (SDS:urea:buffer final ratio 1:1:1 v/v). The samples were then incubated at room temperature for 10 min and applied to a 10% acrylamide gel. Following electrophoresis, the bands corresponding to the α- and β-subunits were cut out, and the protein eluted overnight from the gel pieces into 0.5 mL of water. The gel pieces along with the eluates were placed in cuvettes, and the fluorescence (of CPM) was measured at 466 nm (excitation wavelength 395 nm). The level of fluorescence (and hence the extent of CPM modification) could then be compared in the experimental samples.

RESULTS

The present work was initiated in an attempt to determine whether the previously reported protective effect of K and Rb ions against the reduction of S–S bridges in the β-subunit (Kawamura & Nagano, 1984) and against exhaustive proteolytic digestion (Capasso et al., 1992) is a reflection of the functional role of the β-subunit in stabilization or formation

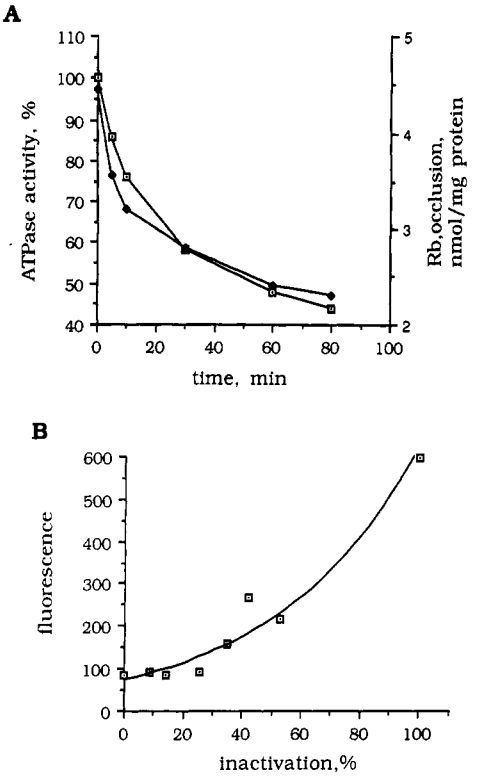

FIGURE 1: Na,K-ATPase [average specific activity of 24–26 μmol of P_i (mg of protein)$^{-1}$ min^{-1}] at 1 mg/mL was incubated in the presence or absence of 0.25 M DTT at 40 °C in 50 mM imidazole/1 mM EDTA, pH 7.5. Aliquots were withdrawn from the DTT-treated sample at the indicated times, diluted with ice-cold buffer, and sedimented. Samples without DTT (control) were incubated for 80 min and then diluted and sedimented. Pellets were resuspended in 0.1 M Tris-HCl, pH 6.95, and assayed for ATPase activity or Rb occlusion (panel A). Heat inactivation of the control sample did not exceed 15%, which was corrected for in experimental samples. For complete inactivation, samples were heated for an additional 30 min at 50 °C. Pellets were resuspended in 0.1 M Tris-HCl buffer, pH 6.95, and assayed for ATPase activity (□) or Rb occlusion (♦). Fluorescence of the β-subunit (panel B) was determined after modification of the reduced samples with the sulfhydryl-directed reagent CPM, as described under Experimental Procedures.

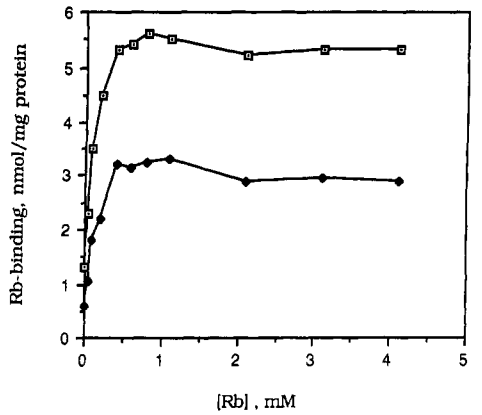

FIGURE 2: Na,K-ATPase, 1 mg/mL, was incubated in the presence or absence of 0.25 M DTT at 40 °C in 50 mM imidazole/1 mM EDTA, pH 7.5. After 1-h incubation, the samples were diluted with ice-cold buffer and sedimented. Pellets were resuspended in 0.1 M Tris-HCl buffer, pH 6.95; aliquots containing of 30 μg of protein were mixed with increasing concentrations of RbCl (2×10^6 cpm of ^{86}Rb per sample) and assayed for Rb occlusion. (□) Control; (♦) DTT-treated sample.

of the ion-binding domain, or whether this ion-specific protection is an indirect effect with no functional significance. We chose DTT or β-mercaptoethanol treatment to study how changes in the extracellular domain, caused by reduction of S–S bridges in the β-subunit, would affect Rb occlusion. It has previously been reported that no reduction of S–S bridges in the α-subunit occurred under our experimental conditions (Kirley, 1990).

DTT Inhibits ^{86}Rb Occlusion by Na,K-ATPase. Incubation of Na,K-ATPase with 0.25 M DTT at 40 °C resulted in time-dependent inactivation of ^{86}Rb occlusion; the rate of loss of ^{86}Rb occlusion is of the same order of magnitude as the rate of loss ATPase activity (Figure 1). It is interesting that loss of occlusion does not proceed beyond about 50% under these conditions, even after washing the enzyme by centrifugation and resuspension followed by a second treatment with the same concentration of DTT. RbCl (20 mM) completely protects against the DTT-induced inactivation of Rb occlusion. More drastic conditions, for example, increasing the temperature and/or raising the DTT concentration (up to 0.5–1 M), resulted in complete inactivation of the Na,K-ATPase. However, inactivation under these conditions seems to be a consequence of the additive effects of increased thermal inactivation, a nonspecific inactivation by DTT (presumably via effects on protein solvation), and specific reduction of S–S bridges. The presence of 5 mM ATP during incubation is without effect on the final level of inactivation of Rb occlusion, but protects the enzyme against heat inactivation. Thus, in the presence of ATP, the of loss of Rb occlusion, the loss of ATPase activity, and the increase in β-subunit SH residues show a clear temporal relationship (Figure 1, panels A and B).

In order to shed more light on the partial (50%) loss of occlusion by the enzyme, the apparent affinity for Rb occlusion was determined before and after DTT treatment. In both cases (Figure 2), half-maximal Rb occlusion was seen around 0.25 mM Rb, and at every concentration of Rb examined, the occlusion following DTT treatment was half of that prior to treatment. This result suggests that the remaining Rb occlusion sites following DTT treatment are essentially unaltered. The treatment apparently knocks out 50% of the sites without affecting the affinity of the remainder. One possible cause of this might be that half of the β-subunits are more sensitive to DTT reduction than the other half.

It should be pointed out that the loss of Rb occlusion is not a nonspecific consequence of any alteration in the structure of the β-subunit. Extensive deglycosylation of the β-subunit with endoglycosidase F at 37 °C causes at most a 5–10% loss in Rb occlusion (data not shown).

The inactivation of Rb occlusion and ATPase activity is accompanied by reduction of S–S bridges in the β-subunit (Figure 1B), which was followed by labeling SH groups with CPM, a fluorescent sulfhydryl-reactive reagent. It can be seen that the relationship between the increase of fluorescence of the β-subunit and inactivation is not linear, illustrating that the three S–S bridges of the β-subunit are not reduced simultaneously. This result is in good agreement with recent data demonstrating the different reactivities of the S–S bridges of the β-subunit toward reducing agents (Kirley, 1990). On the other hand, no more than 1–1.5 additional S–S bridges are reduced when 50% inactivation of Rb occlusion is achieved (see Figure 1B). No changes in the level of fluorescence of the α-subunit were seen. However, the large number of SH groups in the α-subunit and the consequent high level of fluorescence do not allow us to exclude the possibility of reduction of a putative S–S bridge in the α-subunit.

Effects of DTT Treatment on "19-kDa Membranes". To examine more thoroughly the relationship between reduction

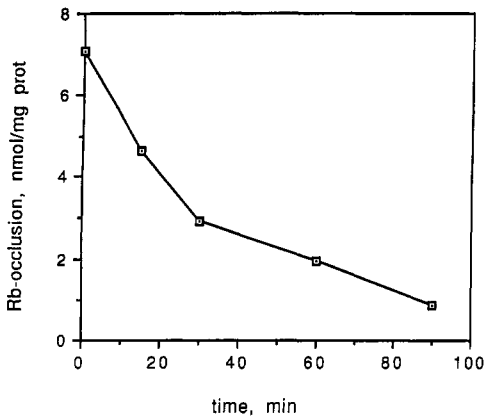

FIGURE 3: Time course of DTT-effected inactivation of Rb occlusion by "19-kDa membranes". "19-kDa" preparation (1 mg/mL) was incubated at 37 °C in 25 mM imidazole, 1 mM EDTA, and 2 mM RbCl in the presence or absence of 0.20 M DTT. Aliquots were withdrawn at the indicated times, diluted with the same ice-cold buffer, and sedimented as described under Experimental Procedures. The loss of Rb occlusion in the absence of DTT was less than 10% over the 90-min treatment period. Pellets were resuspended in the same buffer, and 60 μg of protein was assayed for Rb occlusion.

of the β-subunit and inactivation of Rb occlusion, we took advantage of the posttryptic membrane fraction of Na,K-ATPase (the "19-kDa membranes"). These "19-kDa membranes" can be obtained after extensive proteolytic digestion of Na,K-ATPase in the presence of Rb ions and are composed of predominantly the intramembrane segments H1–H2, H3–H4, and H5, the 19-kDa C-terminal fragment, and an essentially undigested, β-subunit (Capasso et al., 1992). The presence of only nine Cys residues, located in the various postryptic fragments of the α-subunit, simplifies analysis of the structural changes after DTT treatment and enables us to identify that part of the complex, containing S–S bridges, which is critical for integrity of the Rb occlusion domain. These "19-kDa membranes" have been shown to maintain many of the ion-binding characteristics of the native enzyme, and it has been claimed that the 19-kDa C-terminal peptide contains all the essential elements of the cation-binding and occlusion domains of the native enzyme (Karlish et al., 1990). Incubation of these "19-kDa membranes" with DTT (0.20 M) at 37–40 °C resulted in a time-dependent loss of ^{86}Rb occlusion (Figure 3). Because of the instability of the "19-kDa membranes" in the total absence of Rb compared with native enzyme, these experiments were performed in the presence of 2 mM RbCl. Elevation of the RbCl concentration to 25 mM protects the "19-kDa membranes" against 85% of the DTT-produced inactivation. In other words, Rb protection against S–S bridge reduction occurs but is somewhat less effective in the "19-kDa membranes" than in the whole enzyme.

Inactivation of ^{86}Rb occlusion is accompanied by accumulation of a 17-kDa band, when "19-kDa membrane" preparations are separated in a nonreducing Tricine gel (Figure 4A, lanes 2–5, see arrow). The presence of 25 mM RbCl, but not 25 mM choline chloride prevents formation of this band (Figure 4A, lanes 6 and 7). A more precise analysis of the "19-kDa membrane" treated with DTT in the presence and absence of 25 mM RbCl revealed that this 17-kDa band is a doublet (16 and 17 kDa), and both bands were protected by RbCl (not shown). These bands were transferred to a PVDF membrane, and their N-terminal amino acid sequences were determined and shown to be the same: A-K-E-E-G. This primary structure corresponds to a peptide derived from the β-subunit beginning at Ala5. To reveal which components of "19-kDa membranes" were reduced during DTT treatment, samples were labeled with CPM (Figure 4B). No changes in the mobility or the intensity of labeling with CPM were found for the 22-kDa band and the 11- and 8-kDa fragments (Figure 4B), which include the H_1–H_2 and H_3–H_4 transmembrane segments (Capasso et al., 1992). N-Terminal amino acid sequence determination of the 22-kDa band fragment yielded N-P-K-T-D-K-L. Hence, this is the same peptide that was previously reported as the 19-kDa fragment (Capasso et al., 1992). We were unable to detect a 45-kDa fragment of the β-subunit in the membrane-bound fraction (the pellet). However, the 45-kDa fragment derived from the β-subunit after cleavage of the C-terminal 17-kDa segment was isolated in the supernatant. The concentration of 45-kDa fragment in the supernatant increased during the period of DTT reduction and correlated with accumulation of the 17-kDa fragment in the pellet. A correlation was seen between the fluorescent labeling of this fragment and loss of Rb occlusion (Figure 5). Release of this fragment to the supernatant and retention of the remainder of the β-subunit in the membrane pellet confirm the single membrane crossing structure of the β-subunit. No evidence was obtained for other membrane-anchored fragments derived from the β-subunit. This observation is at odds with the earlier result of Capasso et al. (1992), who were unable to remove the 45-kDa fragment by washing the membranes after reduction of S–S bridges with high concentrations of DTT. There are insufficient experimental details in the earlier report to enable us to account for this difference.

Pronase-Digested "19-kDa Membranes". It has been claimed that Pronase digestion of "19-kDa membranes" results in extensive degradation of the β-subunit present in the "19-kDa membranes" and following Pronase treatment (and extensive β-subunit digestion) occlusion is essentially the same as before treatment (Capasso et al., 1992). However, these conclusions were reached following examination of the stained profile of peptides following SDS–PAGE under *reducing* conditions. We have examined the effects of DTT treatment on Pronase-digested "19-kDa membranes". We have found that DTT treatment of these Pronase-treated samples resulted in loss of the Rb occluding capacity which is present prior to DTT-induced reduction. Subsequent treatment of reduced and nonreduced Pronase-digested membranes with CPM and analysis of SDS–PAGE in (nonreducing) 15% Laemmli gels revealed no changes in mobility or fluorescence of the 19-kDa membrane protein bands. It was found that after DTT treatment, a 40-kDa band of the β-subunit disappeared, and several bands with molecular masses of ~13K–15K were produced (Figure 6). The same result was obtained for proteinase K-treated enzyme (data not shown). This suggests that the Pronase-cleaved fragments of the β-subunit remain held together by S–S bridges prior to reduction. It is cleavage of these S–S bridges (by DTT or mercaptoethanol) which results in loss of the β-subunit organization and loss of occlusion. Such a distinction is lost if the analysis is only performed in gels run under reducing conditions.

DISCUSSION

In the present, work, we have obtained evidence for a critical role for the extracellular portion of the β-subunit in stabilizing the occlusion of K^+ ions by the Na pump. The retention of S–S bridges appears to be essential to the organization of the extracellular domain of the β-subunit and hence stabilization of the cation-binding/occlusion domain of the cation-transporting Na,K-ATPase. The central importance of the occlusion of cations in both the enzymatic and transport cycles

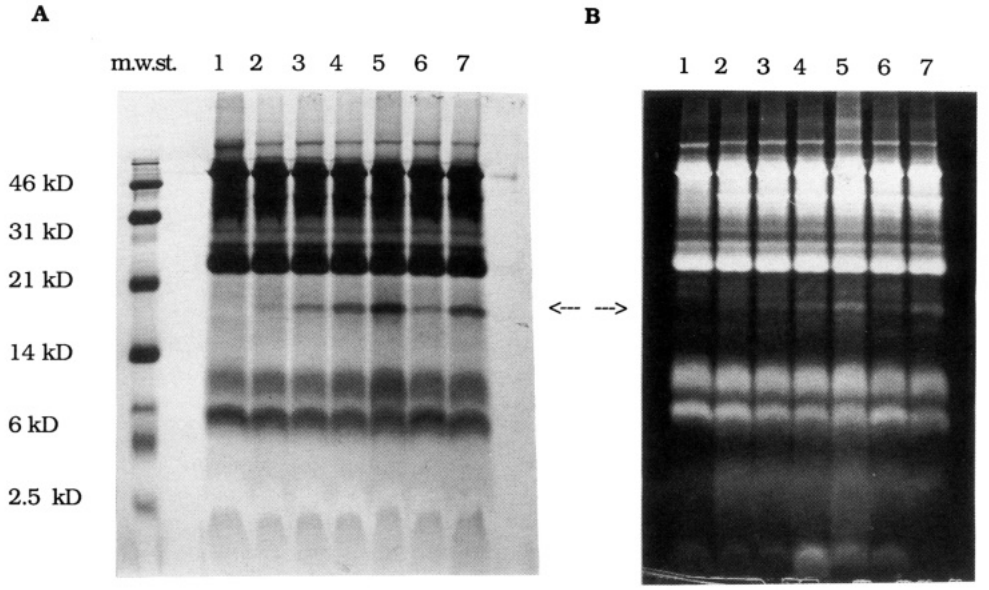

FIGURE 4: Accumulation of N-terminal fragment of the β-subunit along with loss of Rb occlusion. "19-kDa membrane" preparation was treated as in Figure 3. Aliquots of 40 μg of protein were labeled with CPM as described under Experimental Procedures and then separated by SDS–PAGE in a Tricine gel under nonreducing conditions. (A) Coomassie staining; (B) fluorescence. From left to right: low molecular mass standards, control (60 min, no DTT), 15 min, 30 min, 60–min treatment, 60 min followed by 15-min heating at 60 °C, 60 min with 25 mM RbCl, and 60 min with 25 mM choline chloride.

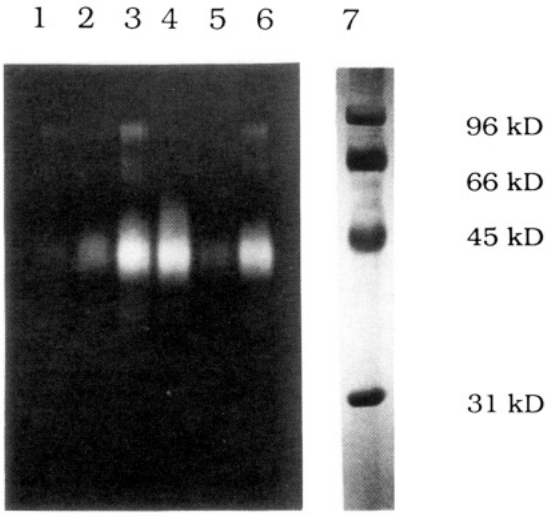

FIGURE 5: Release of the 45-kDa fragment of the β-subunit to the supernatant during reduction of "19-kDa membranes" with DTT. The "19-kDa" preparation was treated as in Figure 3 and 4. Supernatant was treated with CPM as described under Experimental Procedures, and fragments were separated by SDS–PAGE in a 12% Laemmli gel. From left to right: (1) 10 min, (2) 30 min, (3) 90 min, (4) 60 min, (5) 60 min + 25 mM RbCl, (6) 60 min + 25 mM choline chloride, and (7) molecular mass standards.

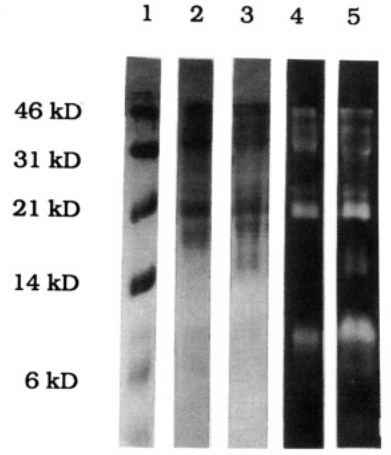

FIGURE 6: The "19-kDa membrane" preparation was digested with Pronase and then treated in the absence of DTT (lanes 2 and 4) or in the presence of 0.20 M DTT (lanes 3 and 5) at 37 °C for 30 min. Samples were labeled with CPM and separated by a Tricine gel under nonreducing conditions. (1) Molecular mass standards; (2, 3) Coomassie staining; (4, 5) fluorescence.

has been discussed previously (Post et al., 1972; Kenney & Kaplan, 1988; Glynn & Karlish, 1990).

Effects of S–S Reduction of Native Enzyme. We found that incubation of Na,K-ATPase with 0.25 M DTT at 40 °C resulted in 50% inactivation of Rb occlusion, which correlates with partial reduction of the β-subunit. It is interesting that 50% of the Rb occluding capacity of the enzyme is abolished. A second similar treatment causes no further loss. This result suggests that there must be two populations of β-subunits with differing sensitivity to reduction by DTT. The source of this heterogeneity is not known; however, one possible basis may be in organization of the α- and β-subunits of the Na,K-ATPase into dimers, where the organization of the dimers renders one of the pair of β-subunits more susceptible to DTT treatment. Mechanistic evidence suggests that if such functional dimers exist, each monomer would have to act out of phase with the other. Experiments to explore these findings are currently underway.

It is also interesting that the residual sites, following DTT treatment, have an unaltered affinity for Rb (Figure 2). Two possible reasons for this are (i) either all of the Na,K-ATPase molecules can now only bind one Rb ion (compared with two ions prior to reduction) (ii) or half of the Na,K-ATPase molecules in the preparation are unaffected by DTT treatment. Since the 50% loss of Rb occlusion is associated with degradation of only part of the total population of β-subunit, we prefer the second of these two explanations. It is not clear why such high concentrations (250 mM) of DTT are required to obtain the effects we observed. However, while this paper was in preparation, a similar report appeared on a closely related protein, the gastric H,K-ATPase. In that work, evidence is provided for reduction of S–S bridges in the β-subunit which leads to a loss of enzyme activity (Chow et al., 1992). In that work, similarly high concentrations of DTT

or mercaptoethanol were also required. It is possible that such high concentrations are required because of the compactness and inaccessibility of the S–S bridges or that they are rapidly reoxidized (by aerial oxidation) following reduction.

Studies with "19-kDa Membranes". Extensive tryptic digestion of the native Na,K-ATPase produces a residual family of membrane-embedded peptides. These membranes contain a 19-kDa C-terminal fragment of the α-subunit (which we find often has an apparent molecular mass of 22 kDa), fragments of the other transmembrane domains of the α-subunit, and a largely undigested β-subunit. Such membranes have been recently shown to occlude Na^+ and K^+ ions in a similar fashion to native membranes, and the 19-kDa component of the peptide mixture has been identified with the cation occluding domain of the native Na,K-ATPase (Karlish et al., 1990). Since the peptide composition of these membranes is simpler than the native enzyme, we decided to investigate the effects of DTT treatment on the ability of the "19-kDa membranes" to occlude Rb^+ ions. The "19-kDa membranes" show a similar sensitivity to DTT treatment, and after S–S bridge reduction, a major part of the occlusion capacity is lost. Instability of 19-kDa membranes to heat and poorer protection by RbCl against inactivation (compared with native enzyme) point to the involvement of other domains in the native enzyme which may not be directly involved in ion occlusion but stabilize this structure.

The incubation with DTT (and loss of Rb occlusion) is correlated with the appearance of a 17-kDa peptide fragment in SDS–PAGE (Figure 5A,B) and the release of a 45-kDa fragment to the supernatant after centrifugation of the DTT-treated membrane. The appearance of the 17-kDa fragment and its identification as beginning at Ala5 of the β-subunit confirm that in producing the "19-kDa membrane" (in addition to a small N-terminal cleavage) the β-subunit is split between the components of an S–S bridge, which is subsquently cleaved by DTT, releasing the 16- and 17-kDa tryptic fragments. [Complete reduction of the S–S bridges in the presence of SDS revealed that only some of the β-subunits were digested by trypsin. This partial digestion at pH 7.5 was observed by Caspasso et al. (1992), who also determined the position of cleavage.] There is such a tryptic cleavage site at Arg140–Gly141 of the β-subunit which is between Cys125 and Cys148 (Kirley, 1989). Identical N-terminal amino acid sequences of the 16- and 17-kDa fragments and their stoichiometry indicate that additional tryptic cleavage occurs, possibly at Lys133–Glu134 or more likely at Arg135–Gly136; both are located within this first S–S bridge. This S–S bridge has been previously shown to be readily reduced by DTT (Kirley, 1990). Thus, the accumulation of the 17-kDa fragment is a good indicator of β-subunit reduction by DTT. These structural data and the correlation with the loss of Rb occlusion point to the critical importance of the β-subunit S–S bridge in the extracellular domain stabilizing the occlusion of transported cations. It was previously reported that the DTT effect on ATPase activity could not be associated with the reduction of one particular S–S bridge (Kirley, 1990). Further experiments are needed to specifically reduce S–S bridges in the β-subunit and to examine whether reduction of any single S–S bridge is sufficient for inactivation or whether extensive reduction of all of the S–S bridges is required.

One may argue that instead of specifically affecting Rb binding, DTT treatment destroys the tetriary structure of the β-subunit and loss of the α and β interactions resulted in conformational changes of the α-subunit, which are lethal for all of enzyme function. If this were the case, the role of the extracellular domain of the β-subunit is to provide an appropriate conformation for the α-subunit; this appropriate conformation remains prese ved even in posttryptic membranes. Our results do not allow us to definitively choose between this rather "nonspecific" disruption of secondary structure and "specific" changes to the ion-binding domain. It is obvious, however, that Rb binding cannot be achieved without the normally folded β-subunit extracellular domain. If the β-subunit was not important for ion occlusion (and transport), as had been suggested previously, reduction of the β-subunit S–S bridges would not be expected to affect cation occlusion.

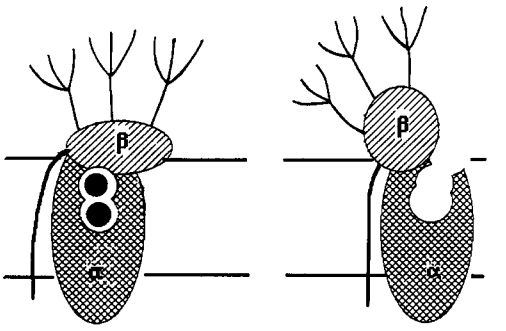

FIGURE 7: Sketch of the β-subunit interaction with the α-subunit in the occluded cation state (left) and in the absence of occlusion (right).

Other data also point to participation of the β-subunit in conformational changes related to the K-binding process. It is well-known that binding of ATP stabilizes the Na,K-ATPase against detergent inactivation, which involves dissociation of subunits and changes in their secondary structure. In the case of reduction of the β-subunit, ATP has no effect, but Rb protects the enzyme very effectively and specifically against inactivation and reduction (Kawamura & Nagano, 1989; this paper). Moreover, Rb specifically (Rb > Na, choline has no effect) protects posttryptic "19-kDa membranes" against DTT inactivation and the β-subunit against reduction. A profound protective effect against DTT reduction is seen with Mg in the presence of ouabain, but not with Mg and P_i (unpublished observation). This suggests that forming an E_2-like conformation is not sufficient to produce protection against reduction. It appears that reagents interacting with the Na pump at the extracellular surface and locking a Rb-bound-like conformation at the same time have effects on the β-subunit. The accumulation of the β-subunit 45-kDa fragment in the supernatant after DTT treatment of "19-kDa membranes" confirms the existence of only one N-terminal transmembrane fragment in the β-subunit, so that participation of the 45-kDa fragment in formation of the transmembrane ion pathway can be excluded. The high concentration of the reducing reagent (DTT) which was necessary to cleave S–S bridges and modify the β-subunit extracellular region points to the existence of a very compact and stable structure. We suggest that the extracellular portion of the β-subunit may serve as a stabilizing "cover" for the ion-binding domain, partially buried in the membrane. It interacts tightly with the intramembrane portion of the Na pump, where Rb or K are bound, and closes access to the K-binding site (Figure 7), also hindering Rb (K) dissociation to the extracellular region. After the phosphorylation step, a structural rearrangement occurs, and the β-subunit changes its conformation in such a way that access can be gained to the ion occlusion site and the β-subunit itself becomes more exposed to reduction and proteolytic degradation. Experiments aimed at obtaining better resolution of the β-subunit spatial organization and finding contact areas

between the β-subunit and α-subunit fragments are now underway.

In summary, we have provided evidence that reduction of β-subunit S–S bridges in the native Na,K-ATPase or in residual posttryptic "19-kDa membranes" results in a significant loss of monovalent cation occlusion. Evidently, the β-subunit plays an important role in stabilizing occluded cation intermediates in the enzyme and transport cycles. The organization of the extracellular domain of the β-subunit, which is maintained by S–S bridges (even after proteolytic cleavage with Pronase), is critical to enzyme function.

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