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Chymotryptic cleavage of α-subunit in E_1-forms of renal $(Na^+ + K^+)$-ATPase: effects on enzymatic properties, ligand binding and cation exchange

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Chymotrypsin in NaCl medium at low ionic strength rapidly cleaves a bond in the N-terminal half of the α-subunit of pure membrane-bound $(Na^+ + K^+)$-ATPase from outer renal medulla. Secondary cleavage is very slow and the α-subunit can be converted almost quantitatively to a 78 kDa fragment. The sensitive bond is exposed to cleavage when the protein is stabilized in the E_1 form by binding of Na^+ or nucleotides. The bond is protected in medium containing KCl (E_2K form), but it is exposed when ADP or ATP are added (E_1KATP form). Fluorescence analysis and examination of ligand binding and enzymatic properties of the cleaved protein demonstrate that cleavage of the bond stabilizes the protein in the E_1 form with sites for tight binding of nucleotides and cations exposed to the medium. About two ^{86}Rb ions are bound per cleaved α-subunit with normal affinity ($K_d = 9$ μM). The bound Rb^+ is not displaced by ATP or ADP. The nucleotide-potassium antagonism is abolished and ATP is bound with high affinity both in NaCl and in KCl media. Na^+-dependent phosphorylation is quantitatively recovered in the 78 kDa fragment, but the affinity for binding of [^{48}V]vanadate is very low after cleavage. ADP-ATP exchange is stimulated 4–5-fold by cleavage; while nucleotide dependent Na^+-Na^+, K^+-K^+, or Na^+-K^+ exchange are abolished. Cleavage with chymotrypsin in NaCl at the N-terminal side of the phosphorylated residue thus stabilizes the E_1 form of the protein and abolishes cation exchange and conformational transitions in the protein although binding of cations, nucleotides and phosphate is preserved. In contrast, cleavage with trypsin in KCl at the C-terminal side of the phosphorylated residue does not interfere with E_1-E_2 transitions and Na^+-Na^+ or K^+-K^+ exchange. This data support the notion that cation exchange and E_1-E_2 transitions are thightly coupled.

Introduction

Controlled proteolysis of the α-subunit of pure membrane-bound $(Na^+ + K^+)$-ATPase in combination with enzymatic studies [1,2], chemical labelling [3–5], and studies of Na^+,K^+-transport in reconstituted vesicles [6–8] have provided valuable information about structure-function relationships and the topology of this protein in the membrane. For these studies it has been important that localization of proteolytic splits within the α-subunit can be controlled with Na^+ and K^+ because transitions between Na-bound forms (E_1Na) and K-bound forms (E_2K) of the enzyme protein are accompanied by changes in the exposure of three different trypsin sensitive bonds on the membrane surface [1,2].

In the present work we examined the enzymatic and transport properties of $(Na^+ + K^+)$-ATPase after chymotryptic cleavage of the α-subunit. We observed that a single bond in the α-subunit is exposed to rapid and selective chymotryptic cleavage in NaCl medium at low ionic strength. Cleavage of this bond was examined as a function of

cation and nucleotide concentrations to see if the variations in rate of cleavage are due to shielding of substrate sites or to the change in conformation of the protein. To study the mechanism of chymotryptic inactivation of $(Na^+ + K^+)$-ATPase activity the binding of cations and nucleotides was examined before and after cleavage. Fluorescence techniques, phosphorylation and cation exchange reactions were performed to compare the effect of chymotryptic and tryptic cleavage on the conformational state and cation transport properties of the cleaved fragments. The study illustrates the usefulness of the technique for selective chymotryptic modification for examining structure-function relationships of the α-subunit, in particular the relationship between structural changes in the protein and cation binding, occlusion and translocation by the Na^+,K^+-pump.

Methods

$(Na^+ + K^+)$-ATPase was purified in membrane-bound form from pig kidney outer medulla by incubation of crude membranes 1.5–2 mg/ml protein with 0.65–0.71 mg/ml SDS in presence of 3 mM $ATPNa_3$/2 mM EDTA/25 mM imidazole (pH 7.5) at 20°C for 30 min followed by centrifugation at 48000 rpm for 2 h at 10°C in a Ti-14 Beckman or Kontron TZT 48.650 zonal rotor. The rotor was loaded with a 15%–40% (w/v) nonlinear sucrose gradient and a cushion of 45 (w/v) sucrose in 25 mM imidazole/1 mM EDTA (pH 7.5) [9]. $(Na^+ + K^+)$-ATPase was recovered from the fractions of the sucrose gradient by 3-fold dilution with 25 mM imidazole/1 mM EDTA (pH 7.5) without ATP followed by centrifugation overnight at 40000 rpm in a Beckman Ti-60 rotor.

Chymotryptic digestion of pure membrane-bound $(Na^+ + K^+)$-ATPase was started by mixing α-chymotrypsin from Sigma (type II, lot C 4129) or Merck (lot 2307) with $(Na^+ + K^+)$-ATPase in a volume of 100–250 μl of 15 mM Tris (pH 7.5) and NaCl or KCl from 10 mM to 150 mM at 37°C. The use of α-chymotrypsin is important, since cleavage with β-, γ- or δ-chymotrypsin was slower and less specific than cleavage with α-chymotrypsin. The rate constants for cleavage of $(Na^+ + K^+)$-ATPase were about 50-fold lower at 170 mM salt than at 20 mM salt. Digestion with chymotrypsin could therefore be stopped by dilution into ice-cold buffer containing 150 mM NaCl, KCl or Tris-HCl. The chymotrypsin inhibitor, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbonate [10] could not be used since it also inactivates $(Na^+ + K^+)$-ATPase.

Sequential cleavage with chymotrypsin in NaCl and trypsin in KCl was started by adding α-chymotrypsin to 25 μg/ml to 12 ml containing 0.5 mg/ml protein of membrane bound $(Na^+ + K^+)$-ATPase in 15 mM Tris-HCl/10 mM NaCl (pH 7.5). After 15 min at 37°C the reaction was stopped by adding KCl to 150 mM and cooling to 0°C. The mixture was centrifuged for 90 min at 50000 rpm. The pellet was resuspended and diluted to 0.5 mg protein per ml in 150 mM KCl/imidazole 25 mM (pH 7.5). Trypsin was added to 30 μg/ml. After 7 min at 37°C the reaction was stopped by adding soyabean trypsin inhibitor to 90 μg/ml. The membranes were collected by centrifugation for 90 min at 50000 rpm, resuspended in 25 mM Tris-HCl/150 mM NaCl, and washed twice to remove KCl. The pellet was resuspended in 25 mM Tris-HCl/150 mM NaCl (pH 7.5).

For assay of $(Na^+ + K^+)$-ATPase, aliquots containing 0.5–1 μg protein were transferred to test tubes containing 130 mM NaCl/20 mM KCl/3 mM $MgCl_2$/3 mM ATP/25 mM imidazole (pH 7.5). After incubation for 1, 3, and 5 min at 37°C the reaction was stopped with SDS in acid and phosphate was determined by the very sensitive ascorbic acid-citrate method as before [11,12]. The $(Na^+ + K^+)$-ATPase activity was calculated from initial velocity curves. The distribution of peptides and fragments remaining in the membrane was examined by electrophoresis in SDS using 5–15% gradient slab gels prepared according to Laemmli [13]. Gels stained with Coomassie blue were scanned in a LKB 2202 Ultrascan laser densitometer.

For determination of phosphorylation of α-subunit and proteolytic fragments, 80–130 μg $(Na^+ + K^+)$-ATPase protein was incubated at 0°C for 6 s in 3 ml 20–30 μM [γ-^{32}P]ATP (65000 cpm/pmol)/3 mM $MgCl_2$/0–20 mM NaCl/20 mM Tris-HCl (pH 7.5) as before [14]. The reaction was stopped with 3 ml 8% perchloric acid/1.2 mM ATP/1.2 mM P_i. The protein was separated by centrifugation and washed two times with 3 ml 4%

perchloric acid/0.6 mM P_i/0.6 mM ATP. The sediment was kept on an icebath and dissolved in 80 μl 1% SDS/10 mM dithiothreitol/50 mM sodium phosphate (pH 2.4). One drop of 40% glycerol and 5 μl 360 μg/ml pyronin Y were added. The phosphoproteins were separated by electrophoresis at pH 2.4 in gradient slab gels (5–15% acrylamide plus bisacrylamide with 3.6% crosslinker). The gels were formed in 1% SDS, 50 mM sodium phosphate (pH 2.4) using 1 mg/ml ascorbic acid, 2.5 μg/ml $FeSO_4$ and 0.003% H_2O_2 for catalysis of crosslinking [15]. After electrophoresis for 3–4 h and 7–9 cm migration of the pyronin dye front, the lanes of the slab gels were cut out, sliced (3 mm), and counted by Cerenkov irradiation in a Packard scintillation counter. In estimates of apparent molecular weights pyruvate kinase (M_r = 57000), lactate dehydrogenase (M_r = 33000) and trypsin inhibitor (M_r = 21000) were used for standardization.

For reconstitution into phospholipid vesicles the ($Na^+ + K^+$)-ATPase was solubilized in cholate, and inserted into phospholipid vesicles by the freeze-thaw technique [16]. Exchange of external media was done by passage of vesicles over Sephadex G-50 [6,17]. The concentration of phospholipid in the vesicle suspensions was determined by fluorescence of diphenylhexatriene [18]. Prior to cleavage of reconstituted enzyme with chymotrypsin the vesicles were centrifuged at 1000 rpm for 5 min through 2 ml syringes containing Sephadex G-50 equilibrated with 10 mM NaCl/25 mM Tris-HCl/1 mM Tris-EDTA (pH 7.0). Chymotrypsin was added to a concentration of 25 μg per 100 μg protein and the vesicles were incubated for 10 min or 20 min at 37°C. Digestion was stopped by passing the vesicles over Sephadex G-100 equilibrated with 10 mM NaCl/22 mM Tris-HCl/3 mM $MgCl_2$. For assay of $^{22}Na^+$-Na^+ exchange, 2 mM [^{14}C]ADP/1 mM ATP and ^{22}Na (5000–15000 cpm/μl) were added. After incubation for 1, 3, or 5 min, the reaction was stopped by boiling for 2 min. For separation of nucleotides, 25 μl medium were transferred to 20 cm polygram gel 300 polyethyleneimine impregnated plastic sheets (Merck). After drying for 5 min the plates were extracted with methanol and developed in 1.2 M LiCl. The nucleotide spots were detected under ultraviolet light, cut out with scissors and eluted in 3 ml 700 mM $MgCl_2$, 25 mM Tris-HCl [19].

Procedures for determination of binding of [^{14}C]ADP [9], [^{48}V]vanadate [12] and ^{86}Rb binding [12] at equilibrium were as described before. Determination of trinitrophenyl-ATP binding [20] and measurement of fluorescence from intrinsic tryptophan [21,22] or fluorescein [23,24] were made in a Perkin Elmer MPF 44 A spectrofluorimeter as described before.

Results

Chymotryptic inactivation of ($Na^+ + K^+$)-ATPase and cleavage of the α-subunit

At low ionic strength, 10–20 mM salt, the Na-bound form of membrane bound ($Na^+ + K^+$)-ATPase was rapidly inactivated by α-chymotrypsin while the K-bound form was resistant to cleavage. As shown in Fig. 1, monoexponential decays were always observed and the ($Na^+ + K^+$)-ATPase and K^+-phosphatase activities were lost in parallel. This is contrast to the dissociation between inactivation curves for ($Na^+ + K^+$)-ATPase and K^+-phosphatase in trypsin medium [2]. In Tris-HCl medium the inactivation rates were intermediate between the rates in NaCl and KCl media. The rate of inactivation of ($Na^+ + K^+$)-ATPase was strongly dependent on ionic strength. As shown in Fig. 2, the inactivation rate constants were reduced by about 50-fold by raising the NaCl concentration from 10 mM to 150 mM. At high salt the rate of inactivation was only slightly higher in NaCl than in KCl medium. The effect of increasing the ionic strength was more pronounced than that previously demonstrated for trypsin [1]. It is due to salt effects on the membrane bound ($Na^+ + K^+$)-ATPase, since the proteolytic activity of chymotrypsin towards casein was independent of ionic strength (not shown).

To examine the nature of these changes in rate of cleavage we studied the effects of gradually exchanging NaCl or KCl for Tris-HCl or of titration with nucleotides. The data in Fig. 3 shows that the addition of NaCl increased the rate constant for inactivation with relatively low apparent affinity, $K_{1/2}$ = 2 mM, while KCl, with $K_{1/2}$ below 100 μM, decreased the rate of inactivation. Addition of ADP increased the rate of inactivation in KCl medium with low apparent affinity, $K_{1/2}$

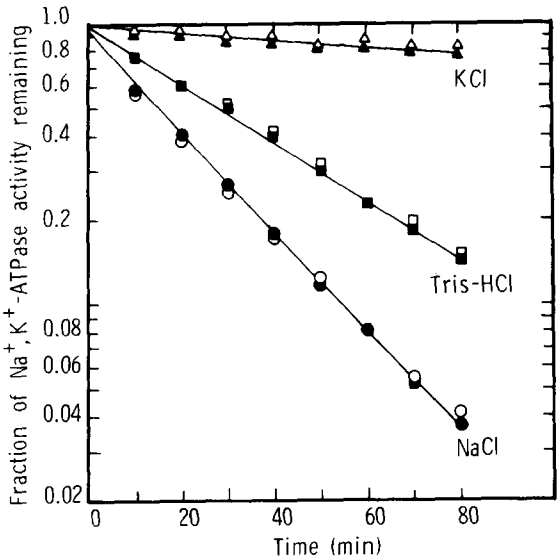

Fig. 1. Effect of NaCl and KCl on chymotryptic inactivation of $(Na^+ + K^+)$-ATPase (closed symbols) and K^+-phosphatase (open symbols) at low ionic strength. Digestion at 37°C was started by adding 1.25 μg α-chymotrypsin to 25 μg $(Na^+ + K^+)$-ATPase in a volume of 250 μl, 15 mM Tris-HCl (pH 7.5) and 10 mM NaCl (●, ○), 10 mM KCl (▲, △) or 25 mM Tris-HCl (■, □). At the indicated times, 10-μl aliquots containing 1 μg $(Na^+ + K^+)$-ATPase protein were transferred for assay of $(Na^+ + K^+)$-ATPase or K^+-phosphatase [12]. The reaction was stopped after 2 and 4 min and the enzyme activities were calculated from linear curves of initial velocity.

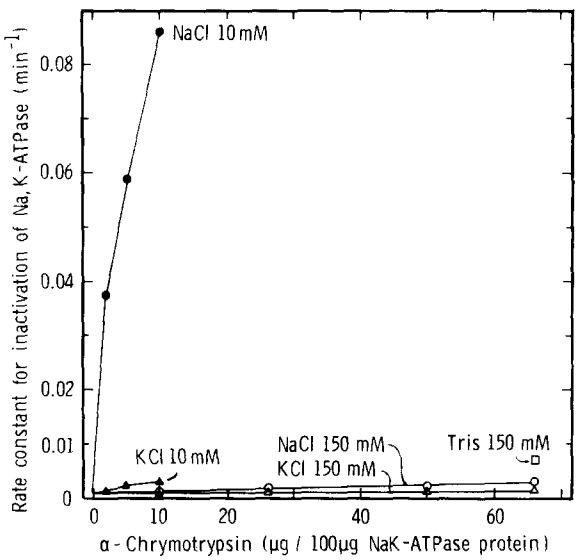

Fig. 2. Concentration dependence of the chymotryptic inactivation of $(Na^+ + K^+)$-ATPase activity at low or high ionic strength in media containing 10 mM NaCl (●), 150 mM NaCl (○), 10 mM KCl (▲), 150 mM KCl (△) or 150 mM Tris-HCl (□). Cleavage was started by adding chymotrypsin and terminated after 30 min at 37°C by 4-fold dilution into icecold 15 mM Tris-HCl (pH 7.5). Aliquots containing 0.7 μg $(Na^+ + K^+)$-ATPase protein were transferred for assay of $(Na^+ + K^+)$-ATPase.

$= 120$ μM ADP, while ADP with $K_{1/2} = 2$ μM reduced the rate of cleavage in NaCl medium to that seen in Tris-HCl (not shown).

The differences in apparent affinity for cations seenin Fig. 3 are similar to previously observed differences in affinities for the effect of the cations on the conformational equilibrium of the protein [1,22,24]. The effect of ADP in KCl medium can be explained by the increase in rate constant of the transition from E_2 to E_1 and stabilization of the ternary complex E_1K-ADP. The straightforward explanation for the data is therefore that the differences in exposure of the bonds to chymotrypsin reflect stabilization of the alternative conformations, E_2K and E_1Na or E_1K-ADP.

Laser densitometry of the gradient slab gels in Fig. 4 shows that the inactivation of $(Na^+ + K^+)$-ATPase in NaCl medium was parallel to cleavage of the α-subunit to a fragment with apparent M_r 78 000. Secondary cleavage was negligible and it is seen that the α-subunit could be converted almost quantitatively to the 78 kDa fragment. In KCl medium the very low rate of inactivation was accompanied with cleavage into fragments with molecular masses close to those arising by tryptic cleavage, i.e. 46 000 and 58 000 [1,2]. Addition of ADP or ATP to the KCl medium led to cleavage of the α-subunit in a pattern similar to that observed in NaCl medium (not shown).

Ligand binding after chymotryptic cleavage

For examination of the effect of chymotryptic cleavage on ligand binding, a batch of $(Na^+ + K^+)$-ATPase was cleaved to 10–20% $(Na^+ + K^+)$-ATPase activity remaining, stored at −80°C in small aliquots and used in a series of ligand binding studies. The experiments in Fig. 5 examines the effect of chymotryptic cleavage on equilibrium binding of ADP, Rb^+, and vanadate. It is seen that the capacity for high affinity nucleotide binding remained unchanged, but the split caused a moderate decrease in the affinities for [^{14}C]ATP

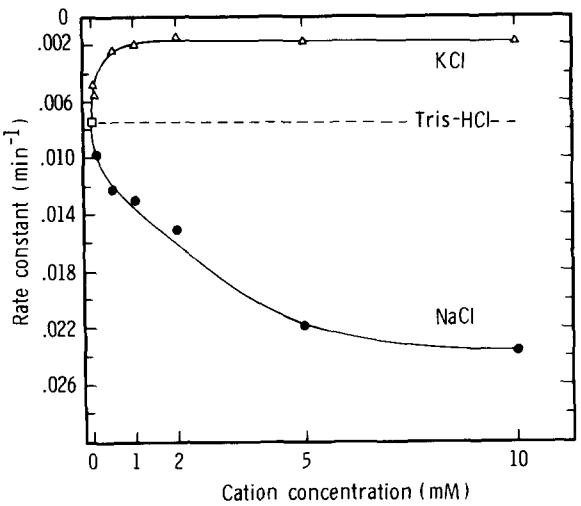

Fig. 3. Titration of the effect of NaCl (●) and KCl (△) on the rate of chymotryptic inactivation of $(Na^+ + K^+)$-ATPase at low ionic strength. Procedure as in Fig. 2 and incubation with 1.25 μg α-chymotrypsin per 25 μg $(Na^+ + K^+)$-ATPase protein and incubation for 20 min at 37°C. Tris-HCl was replaced by NaCl or KCl at constant ionic strength.

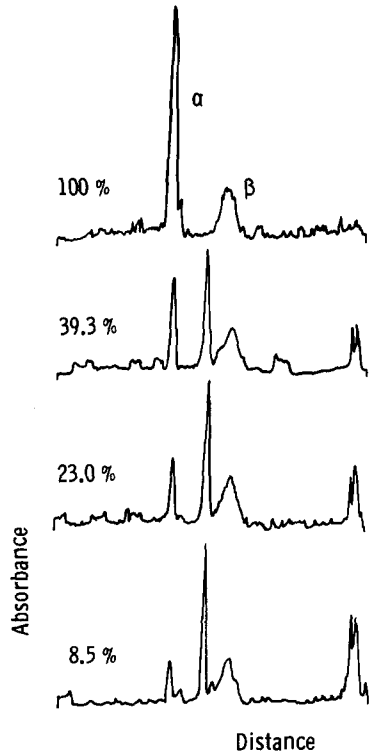

Fig. 4. Formation of 78 kDa fragment by chymotryptic cleavage of the α-subunit of pure membrane bound $(Na^+ + K^+)$-ATPase in NaCl. The figures to the left at each laser densitometry scan indicate the percent $(Na^+ + K^+)$-ATPase activity remaining after incubation with α-chymotrypsin 5 μg per 100 μg protein in 15 mM Tris-HCl (pH 7.5) 10 mM NaCl. After 5 min, 10 min, 20 min or 30 min at 37°C aliquots containing 20 μg $(Na^+ + K^+)$-ATPase protein were suspended in 2% SDS/1% mercaptoethanol/25 mM sodium phosphate (pH 7.0) and boiled for 2 min at 100°C. The samples were applied on 5–15 T% gradient gels prepared according to Laemmli [13]. After staining with Coomassie blue the gels were scanned at 633 nm with resolution < 50 μm in an LKB 2202 Ultrascan laser densitometer using an absorbance range of 0.5.

and [^{14}C]ADP, while the affinity for binding of TNP-ATP was unaffected by the cleavage, Table I. The split did not alter the affinity or capacity for binding of Rb^+ or K^+ as ^{86}Rb was bound with the same capacity, 11–12 nmol/mg protein, and dissociation constant, K_d 9–12 μM [12], before and after cleavage by chymotrypsin. In contrast, vanadate binding was almost abolished after cleavage.

Another drastic consequence of cleavage was the abolition of the nucleotide effect on ^{86}Rb binding. It is seen from Fig. 6 that ^{86}Rb binding to chymotrypsin cleaved enzyme was only moderately reduced by ATP in concentrations up to 1 mM, while binding to control enzyme was practically abolished. Comparison of the Scatchard plots in Fig. 6 with the ^{86}Rb binding data in Fig. 5 shows that the 5–100 μM ATP increased the dissociation constant for ^{86}R binding to control enzyme considerably. After cleavage, even 1000 μM ATP had only a small effect on ^{86}Rb binding. Similarly, the effect of cations on nucleotide binding was practically abolished after cleavage. The data in Fig. 7 demonstrate that chymotrypsin-enzyme bound TNP-ATP with high affinity both in 150 mM KCl and in 150 mM NaCl, while control enzyme, as usual, bound TNP-ATP with low affin-

TABLE I

EFFECT OF CHYMOTRYPTIC CLEAVAGE ON NUCLEOTIDE BINDING TO PURE $(Na^+ + K^+)$-ATPase

Binding of [^{14}C]ATP and [^{14}C]ADP as in Fig. 6 and binding of TNP-ATP as in Fig. 7. Cont., control; Chy, chymotryptic cleavage.

	Capacity (nmol/mg protein)		K_d (μM)	
	Cont.	Chy	Cont.	Chy
[^{14}C]ADP	4.5	4.8	0.11	0.45
[^{14}C]ATP	5.2	5.1	0.045	0.075
TNP-ATP	3.4	4.4	0.081	0.111

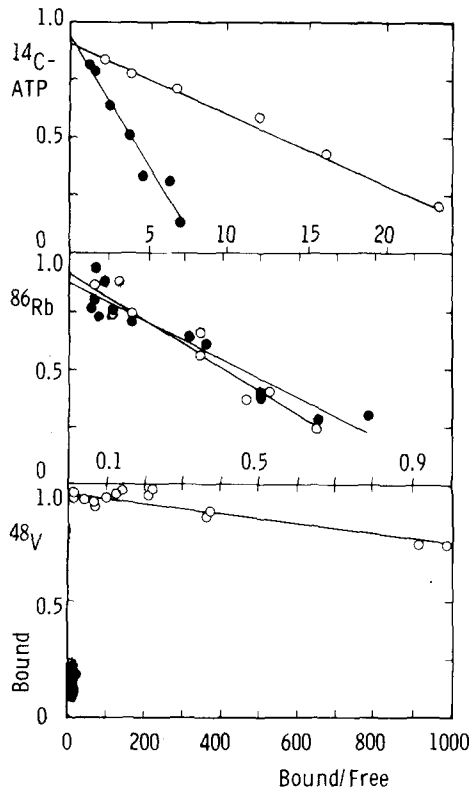

Fig. 5. Equilibrium binding of [^{14}C]ATP (upper panel), $^{86}Rb^+$ (middle panel), and [^{48}V]vanadate to control enzyme (○) or after cleavage with chymotrypsin (●) in NaCl to 18% ($Na^+ + K^+$)-ATPase activity remaining. Binding assay as described before [9,12]. Binding capacities expressed as fraction of binding to the untreated control enzyme.

ity in KCl medium. This means that ATP and Rb^+ were bound simultaneously with high affinity after chymotryptic cleavage and that the potassium-ATP antagonism was abolished without affecting the capacity of the ligand binding sites. This antagonism is usually ascribed to the stabilization of alternative conformations E_2K and E_1ATP by the ligands. As both nucleotide and cation sites were undamaged, the observations therefore support the notion that the cleavage stabilizes E_1 forms of the protein.

Phosphorylation of fragments after sequential cleavage with chymotrypsin and trypsin

The 78 kDa fragment arising by chymotryptic cleavage of the α-subunit in NaCl medium is phosphorylated from ATP in a Na^+-dependent reaction [8]. At 25 μM [γ-^{32}P]ATP, $K_{\frac{1}{2}}$(Na) for phosphorylation of the 78 kDa fragment was 0.7 mM and lower than $K_{\frac{1}{2}}$(Na) of 1.2 mM for phosphorylation of the intact α-subunit [8]. The phosphoenzyme of the 78 kDa fragment was very sensitive to ADP but insensitive to K^+. This shows that chymotryptic cleavage stabilizes E_1 forms of both phospho- and dephospho-forms of the enzyme.

In order to localize the chymotryptic split relative to the phosphorylated residue the fragments receiving phosphate from ATP were identified after sequential cleavage of the α-subunit with chymotrypsin in NaCl and trypsin in KCl medium.

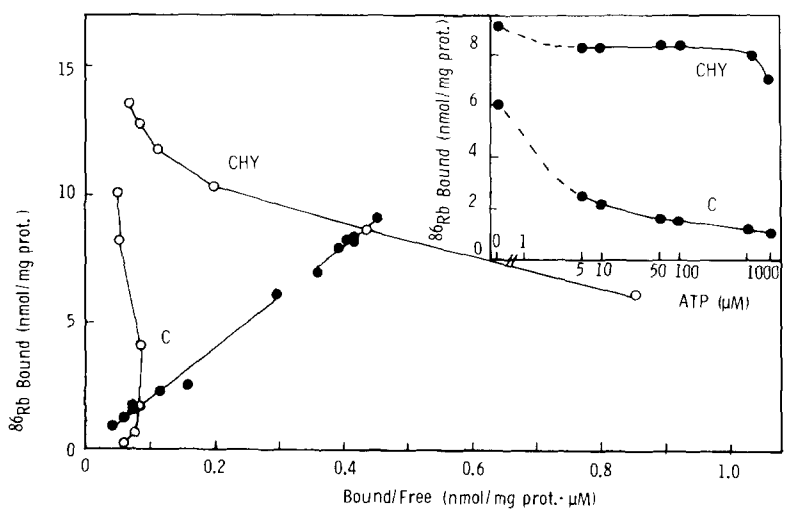

Fig. 6. Effect of chymotryptic cleavage of α-subunit on nucleotide-Rb antagonism. The Scatchard plot (○) shows binding of ^{86}Rb (3.6–202 μM) to control ($Na^+ + K^+$)-ATPase (C) or chymotrypsin-cleaved enzyme (CHY) in the presence of 100 μM ATP. Filled symbols (●) show the effect of varying ATP concentration (0–1000 μM) on binding at 23 μM ^{86}Rb.

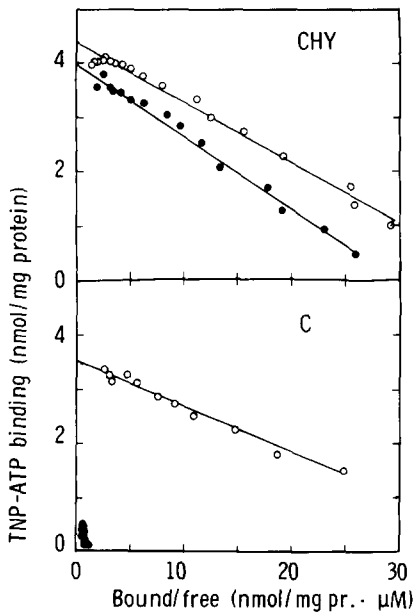

Fig. 7. Binding of TNP-ATP with high affinity in KCl 150 mM (●) and in NaCl 150 mM (○) after chymotryptic cleavage (CHY); while KCl displaces TNP-ATP from untreated ($Na^+ + K^+$)-ATPase (C). Fluorescence titration was carried out with trinitrophenyl-ATP [20] in the range from 0.04 μM to 2.2 μM in a cuvette containing 80–100 μg enzyme protein in 3 ml 25 mM Tris-HCl/0.1 mM EDTA (pH 7.5). Trinitrophenyl-ATP was added from Hamilton syringes with push-button dispensers and the cuvette was thermostated at 10°C. The dissociation constants were for native ($Na^+ + K^+$)-ATPase (C): 0.081 μM in 150 mM NaCl and 3.4 μM in 150 mM KCl and for chymotrypsin-treated enzyme (CHY): 0.11 μM in 150 mM NaCl and 0.13 μM in 150 mM KCl.

In the experiment in Fig. 8, chymotryptic cleavage in medium containing 10 mM NaCl was allowed to proceed until 50% of ($Na^+ + K^+$)-ATPase activity was remaining and about half the α-subunits had been cleaved to 78 kDa fragments (cf. Figs. 1 and 5). Cleavage was stopped by addition of KCl to 150 mM (cf. Fig. 2) and cooling. The membranes were collected by centrifugation and resuspended in 150 mM KCl. Trypsin was added and cleavage was allowed to proceed until ($Na^+ + K^+$)-ATPase activity had again been reduced to 50%. This gave almost equal proportions of four peptides that incorporated ^{32}P from [γ-^{32}P]ATP in a Na^+-dependent reaction, i.e. the intact α-subunit and the 78 kDa, 46 kDa and 18 kDa fragments, Fig. 9. The cleaved enzyme was phosphorylated for 6 seconds at low and high NaCl concentration and the peptides were separated at pH 2.4 in 5–15 T% gradient slab gels that allowed separation of small peptides. The 78 kDa and 46 kDa fragments were formed by direct cleavage of the α-subunit (cf. Ref. 8). It is seen from Fig. 8 that tryptic cleavage of the α-subunit and the 78 kDa fragment produced the 46 kDa and 18 kDa fragments, respectively. Since the tryptic split in KCl is localized to the C-terminal side of the 46 kDa fragment [3], the experiment in Fig. 8 demonstrates that the chymotryptic split in NaCl medium is localized to the N-terminal side of the phosphorylated residue.

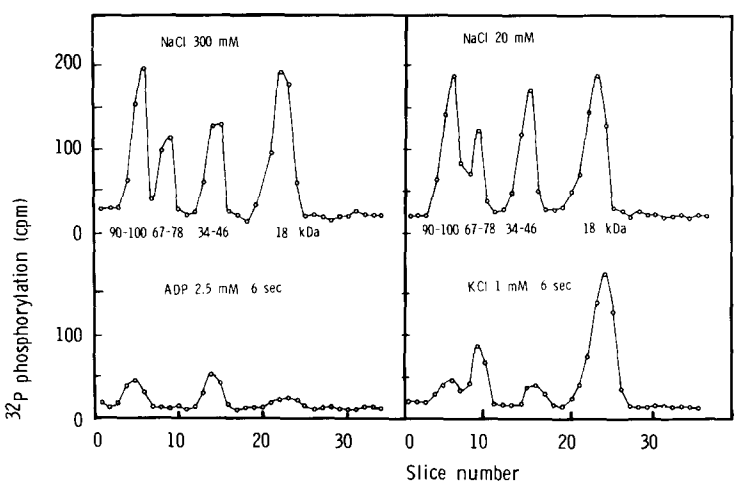

Fig. 8. Phosphorylation and dephosphorylation of intact α-subunit and fragments of α-subunit obtained by sequential cleavage with chymotrypsin in NaCl and trypsin in KCl medium. The enzyme was cleaved as described in Methods and resuspended in 25 mM Tris-HCl/150 mM NaCl (pH 7.5). For phosphorylation 300 μg protein was suspended in 3 ml 25 mM Tris-HCl/3 mM $MgCl_2$/25 μM [^{32}P]ATP/ and 20 mM NaCl (right) or 300 mm NaCl (left). For the samples in the upper half of the graph the reaction was stopped after 6 s with 3 ml 8% perchloric acid. For the samples in the lower half of the graph either 2.5 mM ADP (left) or 1 mM ATP plus 1 mM KCl (right) were added 6 s after start of phosphorylation. The reaction was stopped 2 s later with 3 ml 8% perchloric acid. After sedimentation and wash in 4% perchloric acid the sediment was suspended in SDS and electrophoresed as described in Methods.

Alignment of the phosphorylated peptides is shown in Fig. 9.

Comparison of the reactivity of the four phosphopeptides to ADP and K showed that at 300 mM NaCl, all phosphopeptides were sensitive to ADP, but dephosphorylation of the 78 kDa and 18 kDa fragments was more complete than dephosphorylation of the 46 kDa fragment and the intact α-subunit, Fig. 8. In contrast, at 20 mM NaCl, only the α-subunit and the 46 kDa fragment were sensitive to K^+ while the 78 kDa and 18 kDa fragments were resistant to K^+. Thus after cleavage with chymotrypsin at the N-terminal side of the phosphorylated residue the 78 kDa and 18 kDa fragments accept phosphate from ATP to form ADP-sensitive E_1P, but the two peptides are unable to undergo transition to E_2P. In contrast cleavage by trypsin in KCl at the C-terminal side of the phosphorylated residue does not interfere with transition to E_2P.

Fluorescence responses

Fluorescence from intrinsic tryptophan [21] or the extrinsic probe fluorescein [23,24] was examined to see how cleavage with chymotrypsin would affect the conformational transitions of the protein. As shown in Fig. 10, the cleavage practically abolished the Na^+- or K^+-induced changes in emission from intrinsic tryptophan or from extrinsic fluorescein attached covalently to the α-subunit. Similarly the experiment in Fig. 6 showed that high-affinity binding of TNP-ATP was unaffected by exchanging Na^+ for K^+. Abolition of these responses was not due to insufficient binding of the probes. The capacity for binding of TNP-

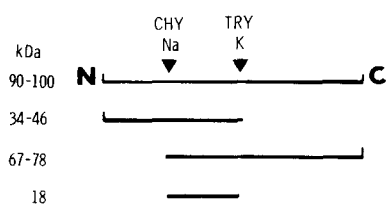

Fig. 9. Alignment of intact α-subunit and fragments receiving ^{32}P from [γ-^{32}P]ATP after sequential cleavage with chymotrypsin in NaCl and trypsin in KCl as in Fig. 8. N and C denote N-terminus and C-terminus of the α-subunit. Based on data in Refs. 2–5 and Fig. 8. The range of molecular masses for the intact α-subunit and the fragment was estimated by standardization as described in Methods.

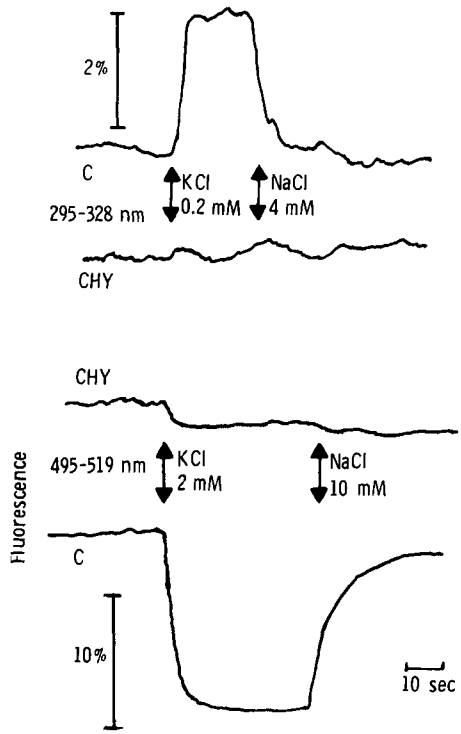

Fig. 10. Cation-induced changes in emission from intrinsic tryptophan or fluorescein attached to the α-subunit in untreated ($Na^+ + K^+$)-ATPase (C) or after chymotryptic cleavage in NaCl (CHY). The cuvettes contained 2.5 ml 25 mM Tris-HCl, 0.1 mM EDTA (pH 7.5) and 25 μg ($Na^+ + K^+$)-ATPase protein. KCl or NaCl were added in portions of 0.5 μl or 1 μl from 1.5 M or 5 M solutions in Hamilton syringes. Tryptophan fluorescence was recorded at 328 nm with excitation wavelength 295 nm. Emission from fluorescein was recorded at 519 nm with excitation wavelength 495 nm using 10 nm slits widths on both monochromators.

ATP was unaffected and the concentration of fluorescein bound to the chymotrypsin-cleaved enzyme was similar to that in control ($Na^+ + K^+$)-ATPase. The results of examining fluorescence responses after chymotryptic cleavage thus confirm the interpretation of the ligand binding and phosphorylation experiments, that the chymotryptic cleavage stabilizes E_1-conformations of the Na^+,K^+-pump protein. It has previously been demonstrated that Na^+- or K^+-induced fluorescence responses are preserved after tryptic cleavage in KCl medium [25].

ADP-ATP exchange and Na^+-K^+ exchange in reconstituted vesicles

In the experiment in Table II we studied the

TABLE II

EFFECT OF CHYMOTRYPTIC CLEAVAGE ON [^{14}C]ADP-ATP EXCHANGE AND Na^+-Na^+ EXCHANGE IN RECONSTITUTED VESICLES

Procedure for chymotryptic cleavage after reconstitution and assay of [^{14}C]ADP-ATP or $^{22}Na^+$-Na^+ exchange as described under Experimental. For assay of active $^{22}Na^+$,K^+-pumping the vesicles were equilibrated overnight with 20 mM RbCl, 15 mM Tris-HCl and passed over Sephadex G-50 to exchange the external medium with 10 mM NaCl/25 mM Tris-HCl/3 mM $MgCl_2$. After addition of ^{22}Na and 3 mM ATP, the vesicles were incubated for 20 s or 40 s at 24°C and passed over columns with Dowex 50-XS [6] and ^{22}Na inside the vesicles was counted. Data are average values of two measurements for each time point.

	Exchange (%)		
	[^{14}C]ADP-ATP exchange	$^{22}Na^+$-Na^+ exchange	$^{22}Na^+$,K^+-pumping
Control	100	100	100
10 min	382	38	35
20 min	498	18	12

effect of chymotryptic cleavage on cation transport and ATP-ADP exchange in reconstituted vesicles. The protein was cleaved after reconstitution to demonstrate the sidedness of the chymotrypsin sensitive bond. Only pumps with the cytoplastic ATP sites facing outward in the reconstituted vesicles can catalyze ATP-dependent Na^+,K^+-pumping and ADP-ATP exchange. The data show that both these reactions are affected by the cleavage. Therefore, the chymotrypsin-sensitive bond must be exposed at the cytoplasmic surface of the Na^+,K^+-pump. The reaction scheme in Fig. 11 shows that the 1 : 1 exchange of Na_{cyt} for Na_{ext} involves transitions between E_1P and E_2P by the forward and backward running of the right hand part of the reaction scheme, while ADP-ATP exchange is catalyzed by E_1 forms of the enzyme.

Chymotryptic cleavage abolished Na^+-Na^+ exchange in parallel to inactivation of Na^+,K^+-pumping, while ADP-ATP exchange was stimulated 4–5-fold, Table II. his shift in equilibrium between E_1 and E_2 forms of the protein with stabilization of the phosphoprotein in the E_1P form explains that Na^+-Na^+ exchange is abolished while ADP-ATP exchange is increased in proportion to the increase in amount of E_1P. Together with phosphorylation and fluorescence data, the results indicate that the chymotryptic cleavage stabilizes the protein in the E_1 conformation and thus prevents transition to E_2.

To determine if chymotrypsin cleavage interfered selectively with translocation of Na^+ or if transport of both Na^+ and K^+ was affected we compared the effects of cleavage on Na^+-Na^+ exchange and K^+-K^+ exchange. The reaction scheme in Fig. 11 shows that ATP-P_i-dependent K^+-K^+ exchange involves transitions between KE_1ATP and KE_2P forms of the protein. In the experiment in Table III, the exchange reactions were examined after cleavage with chymotrypsin

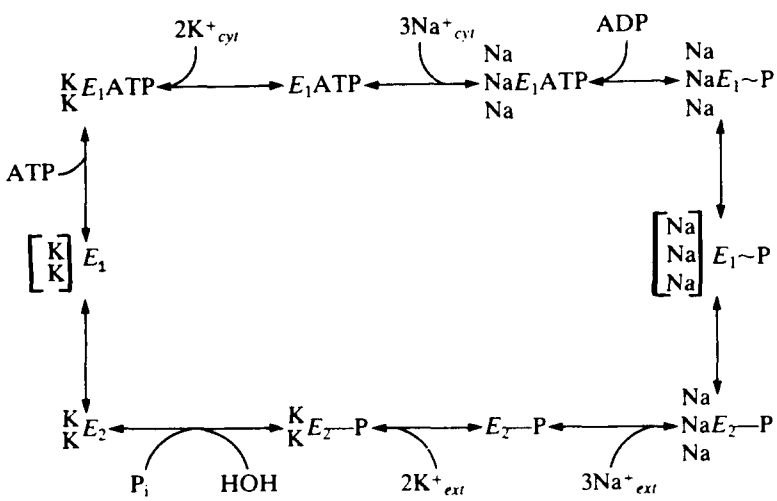

Fig. 11. E_1-E_2 reaction cycle of the Na^+,K^+-pump with ping-pong sequential cation translocation; modified from Ref. 28. Cation sites are exposed to the cytoplasmic surface in E_1 forms. Binding of Na^+ is a condition for transfer of γ-phosphate from ATP to the aspartyl group in α-subunit. Phosphorylation is followed by occlusion of 3 Na^+ per phosphate group. In the E_2 form Na^+-cation sites are exposed to the extracellular medium where Na^+ is released and binding of K^+_{ext} is coupled to hydrolysis of the phosphoenzyme. Dephosphorylation is followed by occlusion of 2 K^+. Uptake of K^+ is associated with E_2-E_1 transition and accelerated by ATP. Na^+ and K^+ within brackets denote occluded ions.

TABLE III

EFFECT OF CHYMOTRYPTIC OR TRYPTIC CLEAVAGE ON ATP-DEPENDENT $^{22}Na^+$-Rb^+ EXCHANGE, ATP-ADP-DEPENDENT $^{22}Na^+$-Na^+ EXCHANGE, AND ATP-P_i-DEPENDENT $^{86}Rb^+$-Rb^+ EXCHANGE IN RECONSTITUTED VESICLES

Membrane-bound $(Na^+ + K^+)$-ATPase was inactivated with chymotrypsin in NaCl as in Fig. 1 to 48% or 33% $(Na^+ + K^+)$-ATPase remaining or with trypsin in KCl to 50% or 30% $(Na^+ + K^+)$-ATPase remaining. Controls were incubated without protease. After solubilization in cholate the enzymes were reconstituted into phospholipid vesicles by the freeze-thaw sonication technique and passed over Sephadex columns to change the outside medium. For $^{22}Na^+$-Rb^+ exchange the intravesicular medium was 100 mM RbCl/25 mM Tris-HCl and the extracellular medium was 10 mM ^{22}Na/110 mM Tris-HCl/1 mM $MgCl_2$/0.2 mM ATP (pH 7.5). For $^{22}Na^+$-Na^+ exchange the intravesicular medium was 110 mM NaCl/25 mM Tris-HCl and the extravesicular medium was 17 mM NaCl/78 mM Tris-HCl/3 mM $MgCl_2$/1 mM ATP/2 mM ADP (pH 7.5). For $^{86}Rb^+$-Rb^+ exchange the intravesicular medium was 25 mM RbCl/100 mM Tris-HCl and the extravesicular medium was 12.5 mM RbCl/102 mM Tris-HCl/4.7 mM P_i-Tris/3 mM $MgCl_2$/0.5 mM ATP (pH 7.5). Incubation times at 20°C were 1, 2, 5 or 10 min and the rates were calculated from linear curves of initial velocities. Exchange rates were calculated as the differences in rate with and without nucleotides in the medium for $^{22}Na^+$-Rb^+ and $^{22}Na^+$-Na^+ exchange at 20°C. The rate of ^{86}Rb-Rb^+ exchange was calculated as the difference in rate with and without 1 mM vanadate in the extravesicular medium. Chy-Try, chymotryptic cleavage; Try, tryptic cleavage. The figures for exchange are expressed in nmol/min per mg protein.

	$(Na^+ + K^+)$-ATPase (%)	ATP $^{22}Na^+$-Rb^+ exchange	ATP-ADP $^{22}Na^+$-Na^+ exchange	ATP-P_i $^{86}Rb^+$-Rb^+ exchange
Control (Na^+)	100	455 (100%)	128 (100%)	168 (100%)
Chy-Try (Na^+)	48	133 ((29%)	59 (46%)	25 (15%)
Chy-Try (Na^+)	33	85 (19%)	19 (15%)	16 (10%)
Control (K^+)	100	380 (100%)	141 (100%)	112 (100%)
Try (K^+)	50	219 (58%)	100 (71%)	124 (105%)
Try (K^+)	30	188 (49%)	71 (50%)	102 (91%)

in NaCl or with trypsin in KCl. In this experiments the enzyme was cleaved before reconstitution, but the results were identical whether the enzyme was cleaved before or after reconstitution. It is seen that chymotrypsin cleavage eliminated not only ADP-ATP-dependent Na^+-Na^+ exchange, but also ATP-P_i-dependent K^+-K^+ exchange. Both modes of cation exchange remained unaffected after cleavage with trypsin in KCl. This is in agreement with the observation that E_1-E_2 and E_1-P-E_2-P transitions are preserved after cleavage by trypsin in KCl. The blocking of both cation exchange and conformational transitions after chymotryptic cleavage demonstrates that the nucleotide-dependent cation-exchange reactions are tightly coupled to the conformational transitions.

Passive cation fluxes after chymotryptic cleavage

The experiments above raises the question whether chymotryptic cleavage interferes with the ability of cation sites to switch between inside-exposed and outside-exposed states. To test this, passive vanadate sensitive Rb^+-Na^+ exchange was assayed before and after cleavage. In this flux mode the Na^+,K^+-pump operates as a carrier

TABLE IV

EFFECT OF CHYMOTRYPTIC CLEAVAGE ON VANADATE SENSITIVE $^{86}Rb^+$-Na^+ EXCHANGE IN ABSENCE OF OTHER LIGANDS

Vanadate-sensitive $^{86}Rb^+$-Na^+ exchange in vesicles reconstituted with pure native $(Na^+ + K^+)$-ATPase (Control) or with chymotrypsin-cleaved enzyme (Chy-Try). Vesicles were formed as in Table III with 20 mM NaCl and 10 mM Tris-HCl (pH 7.0) in the inside medium and the indicated concentrations of ^{86}Rb and Tris-HCl in the outside medium. Incubation for 5, 10, and 20 min at 20°C without and with 3 mM $MgCl_2$, 3 mM $NaVO_3$.

RbCl (mM)	nmol/min per mg protein	
	Chy-Try	Control
0.6	1.2	2.2
3	3.1	5.9
5	3.0	6.2
10	3.6	4.8

catalyzing slow ouabain- or vanadate-sensitive exchange of Na^+ and K^+ in a backwards direction opposite to that of the ATP-dependent Na^+-K^+ exchange [27]. The data in Table IV shows that the passive cation exchange was reduced by 25–52%. The partial preservation of this exchange flux suggests that cation sites retain their ability to switch between inside-exposed and outside-exposed states after chymotryptic cleavage.

Discussion

Chymotryptic cleavage of pure membrane bound ($Na^+ + K^+$)-ATPase is limited to a single bond in the α-subunit and cleavage to a 78 kDa fragment is almost quantitative without secondary hydrolysis. This bond is exposed at the cytoplasmic membrane surface at low ionic strength in conditions where E_1 forms of the protein are stabilized by binding of Na^+ or nucleotides. Chymotrypsin cleaves peptide bonds involving aromatic aminoacid residues. Hydrophobic aromatic residues may therefore be involved in the conformational transition between E_1 and E_2 as previously suggested from tryptophan fluorescence [21] and labeling experiments with iodonaphtylazide [4]. The pronounced sensitivity of this cleavage to changes in ionic strength suggests that the exposure of this bond to chymotrypsin depends on the stability of salt bridges. Protection of the bond at high ionic strength could be explained by reduction of the strength of salt bridges between amino acid and carboxyl groups because the charged groups react with salt ions in solution.

Castro and Farley [3] described cleavage of the chymotrypsin sensitive bond and phosphorylation of the 78 kDa fragment. They localized the split between the NH_2-terminus and the phosphorylation site, but found that cleavage was unaffected by exchange of Na^+ for K^+ in the medium. This variance with our result is readily explained by the pronounced effect of ionic strength on the rate of cleavage and on cation specificity. Castro and Farley worked at physiological ionic strength, where cleavage is very slow and the rate is only slightly higher in NaCl than in KCl medium. The 50-fold increase in rate constant for cleavage of the specific bond following exchange of K^+ for Na^+ is only apparent at low ionic strength (cf. Fig. 2).

This method for chymotryptic modification of the α-subunit provides a valuable tool for establishing structure-function relationships of the Na^+,K^+-pump. The capacities and the affinities of the ligand binding sites remain unaffected, while the cleavage completely blocks ATP dependent Na^+,K^+-pumping and the ($Na^+ + K^+$)-ATPase and K^+-phosphatase activities. Our results show that the explanation for this inactivation is that both dephospho- and phosphoforms of the cleaved α-subunits are stabilized in E_1-forms with cation and nucleotide binding sites exposed at the cytoplasmic membrane surface.

Cleavage and nucleotide binding

The rate of chymotryptic cleavage in NaCl medium is reduced by high-affinity binding of ATP or ADP. These characteristics resemble those described for the exposure of a trypsin sensitive bond in the same segment of the α-subunit to slow cleavage in NaCl medium [1,2]. This suggests that the nucleotides partially protect both the trypsin-sensitive and the chymotrypsin-sensitive bonds by simple shielding when the site is adapted for high-affinity binding in the E_1 form. Both bonds are located about 250 residues away from the N-terminal end of the α-subunit and this segment may be involved in formation of the binding area for nucleotides. Additional evidence for this is that covalent attachment of fluorescein-isothiocyanate (FITC) to the α-subunit reduces the rate of chymotryptic cleavage to levels seen with ADP without altering the location of the split (not shown). FITC binds covalently to a lysine residue in the nucleotide binding area and is known to reduce the rate of cleavage of bond 3 by trypsin [12,30].

Both bonds are completely protected to cleavage by K^+ but they are exposed when ADP or ATP are added to the KCl media. This corresponds to the original description of the exposure of the bonds to trypsin in the media containing the specific ligands [1]. Both the trypsin sensitive and the chymotrypsin sensitive bonds thus participate in the structural changes accompanying transition between the principal conformations of the protein.

Potassium-nucleotide antagonism

The capacity for nucleotide binding is not al-

tered by chymotryptic cleavage. The affinities for binding of ATP or ADP are moderately reduced while that for TNP-ATP remains unchanged, but nucleotide binding is unaffected when Na^+ is exchanged for K^+. Similarly, high-affinity binding of Rb^+ (or K^+) to the cleaved enzyme is unaffected by ADP or ATP.

Chymotryptic cleavage thus abolishes potassium-nucleotide antagonism without significant damage to sites for binding of nucleotides or potassium. The fluorescence changes accompanying transition from E_1Na to E_2K or the reverse transition from E_2K to E_1KATP are also abolished. The data therefore provides evidence for a close correlation between the potassium-nucleotide antagonism and the conformational transition between E_2K and E_1KATP. The demonstration that the cleaved enzyme binds $K^+(Rb^+)$ and ATP simultaneously agrees with our previous conclusion that E_1 forms of $(Na^+ + K^+)$-ATPase exposes high-affinity binding sites for Rb^+ at the cytoplasmic surface [12]. Formation of this ternary complex of enzyme, potassium and nucleotide, E_1KATP, is clearly demonstrated in the cleaved enzyme because the E_1 conformation is stabilized and the transition to E_2 is practically abolished. This complex has been inferred from studies on enzyme that had been treated with modifiers of sulfhydryl groups, *N*-ethylmaleimide [31] or thimerosal [12]. These modifications of sulfhydryl groups also result in a shift in conformational equilibrium in direction of E_1 forms.

In the native Na^+,K^+-pump, high-affinity binding and occlusion of K^+(or Rb^+) occur in parallel with the changes in position of the residues that is monitored by changes in pattern of tryptic cleavage and fluorescence intensities. The equilibrium, E_1K-E_2K, is normally poised in direction of the E_2-form and both high-affinity binding and occlusion of K^+(or Rb^+) therefore appears to be associated with the conformational transition from E_1K to E_2K [32]. The present data shows that the changes in fluorescence intensities accompanying occlusion of Rb^+ or K^+ in the native enzyme are absent after chymotryptic cleavage. Although the chymotrypsin enzyme is stabilized in E_1-forms with respect to configuration of ATP-binding site and fluorescence emission, the cleaved enzyme still binds K^+(or Rb^+) with high affinity and it can occlude K^+(or Rb^+) in a process that is unaffected by ATP [28]. In this occluded form, $E_1(K_n)$, of the cleaved enzyme, both ^{86}Rb binding and occlusion are unaffected by ATP or ADP and the complexes are resolved only after addition of both nucleotide and Na^+.

Our data therefore support the notion that the potassium-nucleotide antagonism depends on the ability of the protein to undergo E_1-E_2 conformational transition, i.e. the stabilization of the alternative conformations E_2K and E_1ATP. The question whether occlusion of K^+(or Rb^+) in chymotrypsin-cleaved enzyme can occur without structural changes that are coupled to the conformational transition between E_1 and E_2 requires further examination.

Cation binding, occlusion and structural changes in protein

Na^+-binding sites are preserved after chymotryptic cleavage since Na^+ stimulates transfer of γ-phosphate from ATP to the protein with higher apparent affinity than in control [8]. The high rate of ADP-ATP exchange and the ADP sensitivity of the phosphointermediate together with disappearance of Na^+-Na^+ exchange show that the cleaved phosphoenzyme is stabilized in the E_1P form. Glynn and co-workers [28] showed that this phosphoenzyme binds 3 Na^+-ions per phosphate molecule incorporated in an occluded form. The operational definition of occlusion is that the cations are not released from the protein during fast passage (< 1 s) through a cation exchange resin [28,32] because exchange of the occluded cation with medium cation is relatively slow.

Chymotryptic cleavage interferes with the motion of segments in the α-subunit that are associated with transition between E_1 and E_2 forms in the native enzyme. Nevertheless the dephosphoform of the cleaved enzyme occludes K^+(or Rb^+) and the phosphoform occludes Na^+ [28]. Together, this data and previous results [12] show that cation sites exposed at the cytoplasmic surface in E_1-forms of both native and cleaved enzyme can bind K^+ (or Rb^+) in the dephosphoform and Na^+ after dephoshorylation from ATP. This process appears to be distinct from the structural changes that are monitored by fluorescence analy-

sis of the E_1-E_2 transition. The present data therefore suggest that binding and occlusion of cation may only involve limited changes in protein structure. The structure of the cation binding sites of the Na^+,K^+-pump is not known, but dicyclocarbodiimide (DCCD) interferes with cation binding [33], suggesting that carbonyl groups form coordinating groups for interaction with the cations. Models developed by Grell and co-workers [34] for formation of cation complexes with ionophores appear to be useful for relating binding and occlusion of K^+ or Na^+ to structural changes that are restricted to the cation binding sites. In this model cation binding sites consist of electrophilic carbonyl groups that are located in a fixed cavity to accomodate the size of the selected cations inside the Na^+,K^+-pump protein. Transition from an open to a closed or occluded configuration of the cation-binding sites involves only limited changes in conformation of carbonyl residues and a substitution of solvent molecules from the inner coordination sphere of the cation. This is a local change in configuration of the cation binding area that may not necessarily be accompanied by changes in intensity of fluorescence emission or in structure of the ATP-binding area.

In the reaction scheme in Fig. 11 it is assumed that the changes between open and closed configuration of the cation binding sites occur while the protein is in the E_1-form as estimated from the tryptic digestion patterns, the fluorescence intensities and the structure of the ATP binding area. The change from open to closed or occluded conformation of the Na^+ sites of the phosphoenzyme must be completed before the protein can undergo transition from $E_1P(Na_n)$ to E_2PNa_n. This transition to the E_2 form is assumed to involve a coordinated shift in orientation of the cation sites and in structure of the ATP binding area. Similarly it is assumed in this reaction scheme that K^+ ions remain in occluded form in the $E_1(K_n)$ form with cation sites oriented towards the cytoplasmic surface and that this complex only resolves after binding of ATP in presence of Na^+ + .

The model in Fig. 12 shows that bond 3 (the chymotrypsin-sensitive bond) and bond 1 (the trypsin sensitive bond) are separated by lipid embedded transmembrane segments. The cleavage points are assumed to be located on loosely structured loops that are protruding at the cytoplasmic surface. Cleavage of bond 1 by trypsin in KCl medium interrupts the association between domains that are engaged in reaction of the aspartyl phosphate group with ATP [8]. This split may interfere with the proper orientation between the aspartyl phosphate residue in the 46 kDa fragment and a domain in the 58 kDa fragment that is engaged in nucleotide binding. Cleavage of bond 3 by chymotrypsin in NaCl medium effectively disrupts the coupling of phosphoryl transfer to cation exchange. A straightforward explanation for this could be that this split interrupts associations between the segment between bond 1 and 3 containing the aspartyl phosphate residue and a cation binding domain that is formed by the segment of the α-subunit between bond 3 and the N-terminus. The data do not provide evidence for location of the cation binding sites, but a similar location in the N-terminal part of the chain has been proposed for the Ca^{2+}-ATPase of sarcoplasmic reticulum [35].

In the native ($Na^+ + K^+$)-ATPase the binding of cations promotes conformational changes in the α-subunit that alter the structure of other areas. The cation binding can be correlated with accompanying changes in proteolytic digestion patterns and intensities of intrinsic and extrinsic fluorescence. These phenomena are absent in chymotrypsin cleaved enzyme although the binding properties of both cation and nucleotide binding sites are unaffected. Cleavage of bond 3 with chymotrypsin prevents the changes in intensities of fluorescence and the accompanying changes in affinity of ATP binding without interfering with cation binding, occlusion and slow passive cation exchange across the membrane. In the model in Fig. 12 it is proposed that a sodium-binding area that is located between bond 2 and 3 in the N-terminal sequence of the α-subunit interacts with the portion of the protein between bond 1 and bond 3 containing the phosphorylation site. Transition from E_1 to E_2 is thought to involve the concerted movement of these two areas. The chymotryptic split alters the protein structure in such a manner that interactions between the two areas are prevented without affecting their individual functions.

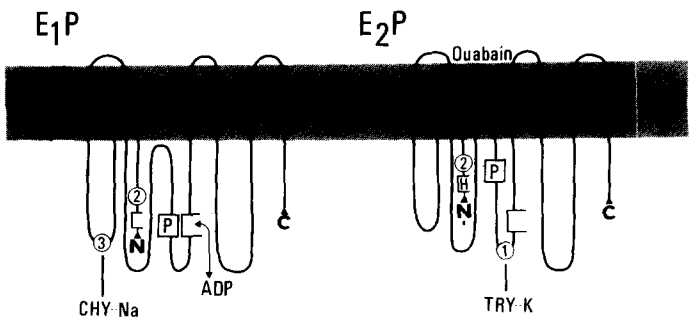

Fig. 12. Model for arrangement of the α-subunit in E_1 and E_2 forms of ($Na^+ + K^+$)-ATPase. Based on data in Refs. 2–5 and Fig. 8. The encircled numbers mark the sites of primary tryptic cleavage in KCl (1) and (2) or NaCl (3) and chymotryptic cleavage in NaCl (3). In the E_1P conformation bonds 2 and 3 are exposed to cleavage while bond 1 is protected. Transition to E_2P is accompanied by protonation of an ionizable group close to the NH-terminus. In the E_2P conformation, bond 3 is protected while bond 1 is exposed to trypsin and the position of bond 2 is such that it is cleaved secondary to cleavage of bond 1 within the same α-subunit. It is proposed that transition from E_1P to E_2P is accompanied by movement by a part of the segment containing the aspartyl phosphate from a relatively hydrophilic to a more hydrophobic environment. The segments between bonds 2 and 3 is proposed to engage in cation binding and formation of the pathway across the membrane. For further explanation see text.

In the native enzyme the fluorescence intensities of the different patterns of tryptic cleavage are assumed to reflect the conformational state of both cation sites and nucleotide binding area. When bond 3 is cleaved the coordinated movement of the two areas is interrupted and the fluorescence changes no longer monitors the switching of sites between inside-exposed (E_1) and outside-exposed (E_2) orientations. Our data therefore suggest that the changes in intrinsic and extrinsic fluorescence accompanying E_1-E_2 transitions in the native ($Na^+ + K^+$)-ATPase reflect motion in the segment of the α-subunit between bond 1 and bond 3.

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