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Conformational Changes of Membrane-Bound $(Na^+ - K^+)$-ATPase as Revealed by Trypsin Digestion

H. Koepsell

Max-Planck-Institut für Biophysik, 6000 Frankfurt (Main) 70, Germany

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Summary. To distinguish ligand-induced structural states of the $(Na^+ - K^+)$-ATPase, the purified membrane-bound enzyme isolated from rat kidneys was digested with trypsin in the presence of various combinations of Na^+, K^+, Mg^{++} and ATP. It was found that first the large and then the small polypeptide chain of the $(Na^+ - K^+)$-ATPase was degraded, indicating that the lysine and arginine residues of the large chain are more exposed than are those of the small one. The $(Na^+ - K^+)$-ATPase activity was inactivated in parallel with the degradation of the large polypeptide chain. After the degradation of the large polypeptide chain, about 75% of the $(Na^+ - K^+)$-ATPase protein remained bound to the membrane, demonstrating that the split protein segments were only partially released.

It was found that the combinations of ATP, Mg^{++}, Na^+ and K^+ present during trypsin digestion influenced the time course and degree of degradation of the $(Na^+ - K^+)$-ATPase protein. The degradations of the large and the small polypeptide chain were affected in parallel. Thus, certain ATP and ligand combinations influenced neither the degradation of the large nor the degradation of the small polypeptide chain, whereas by other combinations of ATP and ligands the degree of susceptibility of both polypeptide chains to trypsin was equally increased or reduced.

In the absence of ATP the time course of trypsin digestion of the $(Na^+ - K^+)$-ATPase was the same, whether Na^+ or K^+ was present. With low ATP concentrations (e.g., 0.1 mM), however, binding of Na^+ or K^+ led to different degradation patterns of the enzyme. If a high concentration of ATP (e.g., 10 mM) was present, Na^+ and K^+ also influenced the degradation pattern of the $(Na^+ - K^+)$-ATPase, but differentially compared to that at low ATP concentrations, since the effects of Na^+ and K^+ were reversed. Furthermore, it was found that the degradation of the small chain was only influenced by certain combinations of ATP, Mg^{++}, Na^+ and K^+ if the large chain was intact when the ligands were added to the enzyme.

The described results demonstrate structural alterations of the $(Na^+ - K^+)$-ATPase complex which are supposed to include a synchronous protrusion or retraction of both $(Na^+ - K^+)$-ATPase subunits. The data further suggest that ATP and other ligands primarily alter the structure of the large $(Na^+ - K^+)$-ATPase subunit. This structural alteration is presumed to lead to a synchronous movement of the small subunit of the enzyme. The structural state of the $(Na^+ - K^+)$-ATPase is regulated by binding of Na^+ or K^+ to the enzyme-ATP complex. The effects of Na^+ and K^+ on the $(Na^+ - K^+)$-ATPase structure are modulated by the ATP binding to "high affinity" and to "low affinity" ATP binding sites.

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Conformational changes of the $(Na^+ - K^+)$-ATPase protein have been studied with different methods [*review* Ref. 14]. One of these, which utilizes the reaction between the purified $(Na^+ - K^+)$-ATPase and specific antibodies to distinguish between conformational states of the enzyme, has been recently applied in our laboratory [14]. Thus, different conformational states which depend on the presence of certain ATP and ligand combinations could be distinguished. It was found that Na^+ and K^+ can alter the antibody inhibition of the $(Na^+ - K^+)$-ATPase – which means, in our interpretation, that Na^+ and K^+ altered the enzyme structure – provided Mg^{++} and ATP were also present. This result is different from data presented by Jørgensen [10, 11], who studied structural states of the $(Na^+ - K^+)$-ATPase by measuring the characteristics of the trypsin degradation of the membrane-bound enzyme and reported that the $(Na^+ - K^+)$-ATPase structure was different when only Na^+ or only K^+ was present.

This discrepancy may be explained by the different methods which were used to detect structural alterations of the $(Na^+ - K^+)$-ATPase and which may each only detect some of the enzyme conformations. As the method employed to remove the nucleotides, present during the $(Na^+ - K^+)$-ATPase preparation, is not described by Jørgensen [9, 10] [11], another explanation could be the presence of tightly bound nucleotides, which in our preparation were completely removed by gel filtration in the presence of glycerol and Mg^{++}. Thus we performed trypsin digestion experiments on the membrane-bound $(Na^+ - K^+)$-ATPase isolated from rat kidneys, including the conditions tested by Jørgensen. Furthermore, the influence of small amounts of nucleotide on the Na^+ and K^+ effects on the trypsin digestion was investigated.

If ATP was completely removed from the $(Na^+ - K^+)$-ATPase, Na^+ and K^+ had no specific effects on the trypsin digestion. If the tightly bound nucleotides, ATP and ADP, remain associated with the enzyme, the Na^+ and K^+ effects, which were described by Jørgensen, could be partially reproduced. Also in the presence of high concentrations of ATP or Mg^{++} plus ATP, Na^+ and K^+ had different effects on the trypsin digestion of the enzyme, but these effects were opposite to those observed in enzyme preparations, which contained tightly bound nucleotides, when no ATP was added.

Materials and Methods

Preparation of $(Na^+ - K^+)$-ATPase

Membrane-bound $(Na^+ - K^+)$-ATPase was purified from the outer medulla of rat kidneys by incubation of a microsomal fraction with sodium dodecyl sulfate and ATP

followed by zonal centrifugation [13]. The specific activity was 30–36 μmol ATP split per mg protein per min, and the purity of the enzyme as checked by sodium dodecyl sulfate gel electrophoresis was about 95%. The adenine nucleotides were removed in the absence and presence of 3 mM $MgCl_2$, by gel filtration on a column of Sephadex G 50 with 50% (vol/vol) glycerol, 20 mM imidazole-HCl, pH 7.5, and 2 mM EGTA as running buffer, or by repeated centrifugation in a buffer containing 25 mM imidazole-HCl, pH 7.5.

Protein and $(Na^+ - K^+)$-ATPase Assay

Protein was determined according to the method of Lowry and coworkers [17] after precipitating the enzyme with 10% trichloroacetic acid and dissolving the precipitate in 1 N NaOH and 10% sodium dodecyl sulfate (SDS) using bovine serum albumin as a standard. The $(Na^+ - K^+)$-ATPase activity was assayed by the pyruvate kinase-lactate dehydrogenase linked system in which hydrolysis of ATP is coupled to the oxidation of NADH [13].

ATP and ADP Content of $(Na^+ - K^+)$-ATPase

$(Na^+ - K^+)$-ATPase preparations, from which the nucleotides had been removed by gel filtration or centrifugation in the presence or absence of $MgCl_2$, were analyzed for their content of ADP and ATP. The nucleotides were extracted from 2 to 4 mg enzyme protein with cold perchloric acid as described by Garrett and Penefsky [4]. ATP was determined by photo counting using the firefly luciferase assay [24] in a Berthold BF 5000 liquid scintillation counter. ADP was assayed after conversion to ATP by the phosphoenolpyruvate-phosphokinase system.

Polyacrylamide Gel Electrophoresis

The two polypeptide chains of the $(Na^+ - K^+)$-ATPase, for which in the rat kidney molecular weights of 120,000 and 60,000 daltons have been determined [13], were identified by SDS polyacrylamide disc gel electrophoresis, which was carried out according to Weber and Osborn [25]. Electrophoresis was run in 0.1 M phosphate buffer, pH 7.4, containing 0.1% sodium dodecyl sulfate. The gels consisted of 8% acrylamide, 0.2% bisacrylamide, 0.06% N,N,N′N′-tetramethylenediamine, 0.1% SDS, 0.035% ammonium persulfate and 0.1 M sodium phosphate, pH 7.1. A constant current of 5 mA per gel was applied for 8 hr. Samples were pretreated with 1% SDS and 1% mercaptoethanol at 90 °C for 10 min. The acrylamide gels were stained in a solution of 0.025 g Coomassie Brillant Blue G 250 and 12.5 g trichloroacetic acid in 100 ml water and destained in 7% acetic acid. The molecular weights of the polypeptide chains were determined by calibration against known standards: RNA, bovine serum albumin, albumin from chicken eggs, cytochrome *c* and human gamma-globulin. The stained gels were scanned at 565 nm. The amount of protein of the $(Na^+ - K^+)$-ATPase subunits was estimated by measuring the areas below the respective peaks on the densitograms.

Trypsin Digestion of the Large Chain of the $(Na^+ - K^+)$-ATPase

To measure the trypsin degradation of the large chain and the simultaneous destruction of the $(Na^+ - K^+)$-ATPase activity of the membrane-bound enzyme, the treatment with

trypsin was carried out at 37 °C with a weight ratio of trypsin to $(Na^+ - K^+)$-ATPase protein of 1:17. The concentration of enzyme protein and trypsin was 0.1 mg ml^{-1} and 6.0 μg ml^{-1}, respectively. During the incubation different combinations of ATP, Na^+, K^+, Mg^{++}, EGTA and choline were present. The cations were added as their chloride salts. After different time intervals after the addition of trypsin (2–90 min) the digestion was stopped by adding soybean trypsin inhibitor to a weight ratio of inhibitor to trypsin of 7.5:1. Samples were immediately measured for $(Na^+ - K^+)$-ATPase activity and aliquots containing 10 or 15 μg $(Na^+ - K^+)$-ATPase protein were taken for the preparation of samples for SDS polyacrylamide gel electrophoresis. Control samples were prepared without trypsin in parallel to each experiment. The decrease in the $(Na^+ - K^+)$-ATPase activity in the controls was less than 5% within 90 min.

Trypsin Digestion of the Small Chain of the $(Na^+ - K^+)$-ATPase

Trypsin treatment of the membrane-bound $(Na^+ - K^+)$-ATPase degrades first the large chain and subsequently the small chain. Thus, the effects of ATP and ligands on the trypsin digestion of the small chain was determined by incubating the enzyme with higher trypsin concentrations and longer time intervals and then analyzing the protein composition of the digested enzyme. Membrane-bound $(Na^+ - K^+)$-ATPase (1 mg ml^{-1} protein) was incubated at 37 °C with trypsin at a concentration of 0.13 mg ml^{-1} in a buffer containing 20 mM imidazole-HCl, pH 7.5, 2 mM EGTA, and varying combinations of ATP, ADP, adenylyl (β-γ-methylene) diphosphonate (AMP-PCP), Na^+, K^+ and Mg^{++}.

Materials

Trypsin from bovine pancreas and soybean trypsin inhibitor (type 1-S) were purchased from Sigma (London). ATP, ADP, and adenylyl (β-γ-methylene) diphosphonate (AMP-PCP) were supplied by Boehringer (Mannheim) and their tris salts were prepared by ion exchange chromatography. Adenosine 5′triphosphate-triethylammonium salt-$\alpha^{32}P$ ([α-^{32}P]ATP) was obtained from New England Nuclear (Boston) and 2–3 H^3adenosine 5′diphosphate-ammonium salt ([^3H]ADP) from the Radiochemical Centre Amersham (Braunschweig). Other chemicals were purchased as described earlier [13].

Results

Removal of Tightly Bound Nucleotides

The nucleotides, ATP and ADP, were removed from the membrane-bound $(Na^+ - K^+)$-ATPase, which had been purified in the presence of ATP, by either repeated centrifugation or by gel filtration in the presence of 50% (vol/vol) glycerol. Both methods yielded similar results. The presence of $MgCl_2$ was essential during the washing or gel filtration in order to completely remove the ADP and ATP (Table 1). In the absence of $MgCl_2$ during gel filtration about 0.4 moles nucleotide per

Table 1. Removal of tightly bound nucleotides from the membrane-bound ($Na^+ - K^+$)-ATPase by gel chromatography in the presence and absence of Mg^{++} [a]

Running buffer	Nucleotides bound to the membrane-bound ($Na^+ - K^+$)-ATPase after passage through a Sephadex G 50 column		
	ADP	ATP	ADP+ATP
	moles nucleotide bound per mole ($Na^+ - K^+$)-ATPase (mol wt 360,000)		
20 mM imidazole-HCl, pH 7.5, 2 mM EGTA 50% (vol/vol) glycerol	0.18 0.21	0.21 0.19	0.39 0.40
20 mM imidazole-HCl, pH 7.5, 2 mM EGTA, 50% (vol/vol) glycerol 3 mM $MgCl_2$	0.02 0.01	0 0	0.02 0.01

[a] Membrane-bound ($Na^+ - K^+$)-ATPase (4 mg protein), purified in the presence of ATP and Na^+, was applied on a Sephadex G 50 column and the nucleotides were separated in the presence and absence of 3 mM $MgCl_2$. The remaining nucleotides were extracted from the ($Na^+ - K^+$)-ATPase with $HClO_4$ [4] and ADP and ATP were determined by the Luciferase method [24].

mole of ($Na^+ - K^+$)-ATPase molecule remained bound to the enzyme[1]. The result that ATP and ADP can be completely removed from the ($Na^+ - K^+$)-ATPase only in the presence of $MgCl_2$, was also obtained when nucleotide-free enzyme was incubated with 2 μM [α-^{32}P] ATP or 2 μM [^{3}H] ADP and then dialyzed against 25 mM imidazole-HCl, pH 7.5. Measuring the time courses of the disappearance of the radioactive nucleotides from the enzyme, it was found that [α-^{32}P] ATP or [^{3}H] ADP were nearly completely removed after 16 hr dialysis in the presence of 3 mM $MgCl_2$ (<1 pmol nucleotide per mg ($Na^+ - K^+$)-ATPase protein). When 10 mM EDTA but no $MgCl_2$ was present the nucleotides were removed much more slowly. The amount of nucleotides which remained bound to the ($Na^+ - K^+$)-ATPase under these conditions was dependent on the final nucleotide concentration in the dialysis bath. At a final nucleotide concentration of 0.2–0.4 nM, 0.2–0.5 nmol nucleotide per mg ($Na^+ - K^+$)-ATPase protein remained bound after 16 hr dialysis.

[1] For this calculation a molecular weight of 360,000 was taken for the ($Na^+ - K^+$)-ATPase, assuming that the enzyme molecule is a tetramer of two large and two small polypeptide chains. However, on the basis of morphological studies on freeze-fractured ($Na^+ - K^+$)-ATPase membranes (*unpublished*) and on the basis of molecular weight determinations in which values up to 500,000 [19] or 670,000 [18] were obtained, it cannot be excluded that the ($Na^+ - K^+$)-ATPase may be a hexamer or an octamer.

Inactivation of ($Na^+ - K^+$)-ATPase activity and Degradation of the Large ($Na^+ - K^+$)-ATPase Subunit by Trypsin

The membrane-bound ($Na^+ - K^+$)-ATPase (0.1 mg/ml) was digested at 37 °C with low concentrations of trypsin (6.0 μg/ml) in the presence of various combinations of Na^+, K^+, Mg^{++} and ATP. The time course of inactivation of the ($Na^+ - K^+$)-ATPase activity was measured. In some experiments the degradation of the 120,000 dalton and of the 60,000 dalton molecular weight subunit was analyzed in parallel. Furthermore, the appearance of some splitting products was observed and the amount of protein released from the membrane during the trypsin treatment was measured.

In the absence of ATP and ligands in the presence of 25 mM imidazole-HCl, pH 7.5, the ($Na^+ - K^+$)-ATPase activity of the nucleotide-free enzyme was inactivated within 15 min to about 3% of the control activity (Fig. 1*a*). In the presence of 10 mM $MgCl_2$ the same time course of inactivation was obtained. If the trypsin digestion was performed in the presence of 10 mM ATP, the inactivation of the ($Na^+ - K^+$)-ATPase activity proceeds more slowly (Fig. 1*a*). However, this ATP effect was not observed if $MgCl_2$ was present in equimolar concentrations. If the variations in ionic strength generated by the addition of 10 mM $MgCl_2$, 10 mM ATP, or 10 mM MgATP were corrected by substitution with choline chloride, the same results were obtained (data not shown)[2]. The time course of enzyme inhibition was the same whether in the absence of ATP, 150 mM choline chloride or 150 mM NaCl or 150 mM KCl was present (Fig. 1*b*). The finding that the trypsin inactivation proceeds more slowly in the presence than in the absence of 150 mM choline chloride (*compare* Fig. 1*a* and *b*) is probably due to an ionic strength effect. If ATP or ATP plus Mg^{++} was present simultaneously with Na^+ or K^+, the time course of trypsin inactivation was different. From Fig. 1*b* it can be seen that the ($Na^+ - K^+$)-ATPase activity was inactivated much more slowly in the presence of Mg^{++}, ATP plus K^+, or ATP plus K^+ than in the presence of ATP, Mg^{++} plus Na^+, or ATP plus Na^+. The time courses of trypsin inactivation shown in Fig. 1*b* are not significantly altered if the differences in ionic strength due to

[2] Measuring the effect of ionic strength on the rate of trypsin inactivation of the ($Na^+ - K^+$)-ATPase activity by varying the concentration of choline chloride during the trypsin digestion, it was found that an increase of ionic strength from 15 to 104 mM did not influence the trypsin digestion. By further increase of the ionic strength, the trypsin inactivation of the ($Na^+ - K^+$)-ATPase activity was slowed down. The main effect was observed in the ionic strength range of 130 to 170 mM.

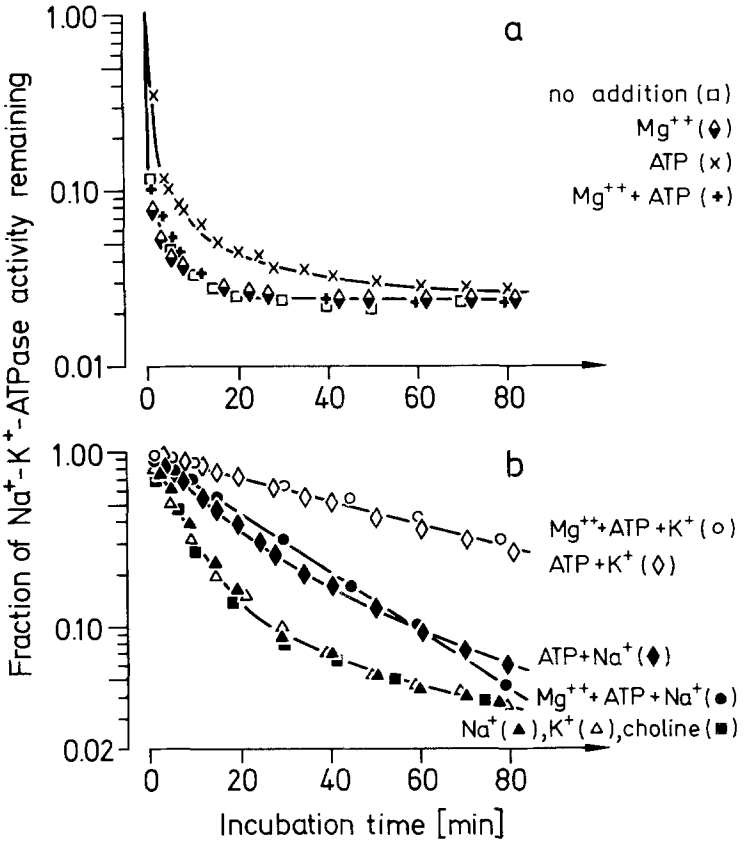


Fig. 1. (*a–b*): Time courses of the inactivation of $(Na^+ - K^+)$-ATPase by trypsin in the presence of different combinations of ATP and ligands. Aliquots of purified membrane-bound $(Na^+ - K^+)$-ATPase containing 200 μg protein were incubated at 37 °C with 12.0 μg trypsin in 2 ml buffer containing 25 mM imidazole-HCl, pH 7.5, and $MgCl_2$ (10 mM), ATP (10 mM), NaCl (150 mM), KCl (150 mM) and choline chloride (150 mM) as indicated in the figure. Digestion was started by addition of trypsin. At the different time intervals 50 μl of the incubation medium was mixed with 25 μl imidazole-HCl, pH 7.5, containing 2.25 μg trypsin inhibitor. Then 50 μl of this mixture was immediately assayed for $(Na^+ - K^+)$-ATPase activity

the addition of ATP and $MgCl_2$ were corrected by the addition of choline chloride. The trypsin inactivation of $(Na^+ - K^+)$-ATPase activity in the presence of Na^+ was significantly reduced if ATP or Mg^{++} plus ATP were also present (Fig. 1*b*). In the presence of ATP plus Na^+ the time course of inactivation was biphasic in the semilogarithmic plot as it was observed under most experimental conditions (Fig. 1*a*, and *b*), whereas a linear relationship was obtained in the presence of Mg^{++}, ATP plus Na^+ as it was also found in the presence of Mg^{++}, ATP plus K^+ or ATP plus K^+ (Fig. 1*b*).

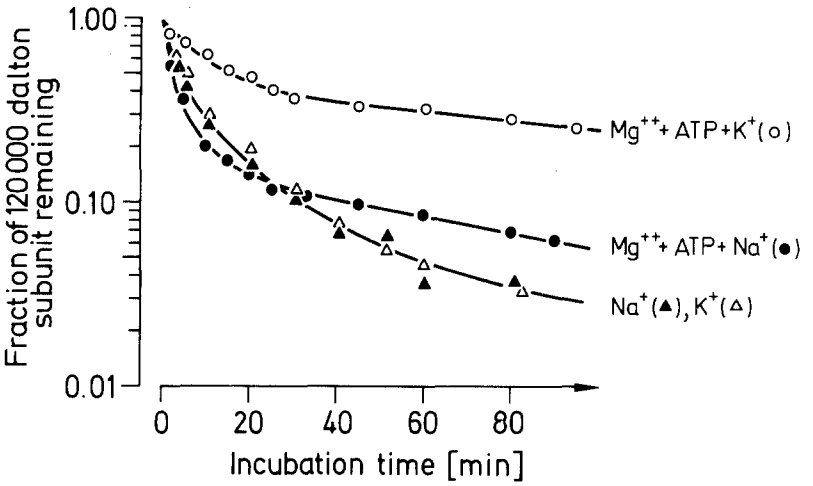


Fig. 2. Time courses of degradation of the large polypeptide chain (120,000 dalton subunit) of membrane bound $(Na^+ - K^+)$-ATPase after trypsin digestion in the presence of some ATP and ligand combinations. Incubation as in Fig. 1 with 200 µg $(Na^+ - K^+)$-ATPase protein and 12 µg trypsin in 2 ml with $MgCl_2$ (10 mM), ATP (10 mM), NaCl (150 mM), and/or KCl (150 mM). After digestion was stopped by mixing with trypsin inhibitor samples were prepared for SDS polyacrylamide gel electrophoresis and the amount of protein of the large polypeptide chain was estimated from the densitograms of the stained proteins as described in *Materials and Methods*

In parallel with the inactivation studies, the effects of Na^+ and K^+ on the time course of digestion of the small and of the large polypeptide chain of the ATP-free, membrane-bound $(Na^+ - K^+)$-ATPase was analyzed. It was found that after incubation with the low trypsin concentration, the small chain protein remained intact for about 80 min if 150 mM NaCl or 150 mM KCl or 150 mM choline chloride was present, whereas more than 95% of the large chain was digested. For the large chain protein it was found analogous to the trypsin inactivation data that the time course of trypsin degradation was different in the presence of Na^+ or K^+ if ATP was also present (Fig. 2). In the presence of ATP, Mg^{++} and K^+, the high molecular weight subunit was degraded much more slowly than in the presence of ATP, Mg^{++}, and Na^+. If the trypsin treatment was performed in the presence of Na^+ or K^+, a biphasic linear relationship between the amount of large chain protein and $(Na^+ - K^+)$-ATPase activity was obtained, whereas a curvilinear relationship was found if the trypsin treatment was performed in the presence of ATP, Mg^{++} plus Na^+ or in the presence of ATP, Mg^{++} plus K^+ (Fig. 3).

Consistent with the findings of Giotta [5], it was found that in the absence of ligands trypsin digestion of $(Na^+ - K^+)$-ATPase revealed a

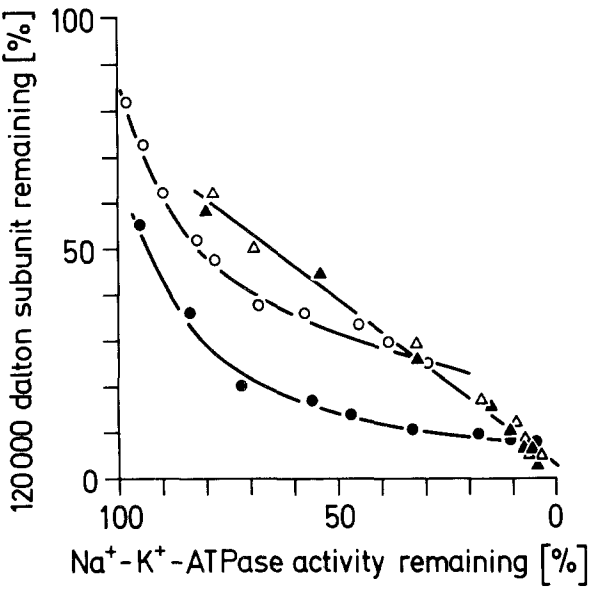


Fig. 3. Relationship between the content of the large polypeptide chain and the activity of the $(Na^+ - K^+)$-ATPase in the presence of 150 mM NaCl (▲); 150 mM KCl (△); 10 mM $MgCl_2$, 10 mM ATP plus 150 mM NaCl (●) or 10 mM $MgCl_2$, 10 mM ATP plus 150 mM KCl (○). Trypsin digestion was performed as described in Fig. 1. At different time intervals after the addition of trypsin samples were taken and the digestion was stopped by mixing with trypsin inhibitor as in Fig. 1. The samples were then either assayed for $(Na^+ - K^+)$-ATPase activity or prepared for SDS polyacrylamide gel electrophoresis. The gels were stained with Coomassie Brillant Blue G 250 and scanned at 565 nm. The amount of protein of the large polypeptide chain was estimated by measuring the areas below the respective peak on the densitograms

transient splitting product with about the same molecular weight as the small $(Na^+ - K^+)$-ATPase subunit, furthermore a more stable 42,000-dalton peptide as well as splitting products with lower molecular weights (data obtained by digestion with a trypsin concentration of 6 μg ml^{-1} are not shown, digestion with a trypsin concentration of 0.13 mg ml^{-1}, *see* Fig. 8*a*). In agreement with Giotta [5], it was further observed that the digestion of the large polypeptide chain proceeded more slowly and that a 90,000-dalton splitting product appeared if the trypsin digestion was performed in the presence of 10 mM ATP (*see,* e.g., Fig. 8*a*). If the trypsin digestion was performed in the presence of 10 mM MgATP the 90,000-dalton peptide was not found (not shown). Similar to the results of Lo and Titus [16] it was observed that also after trypsin digestion in the presence of 150 mM KCl a 42,000 dalton peptide and splitting products with smaller molecular weights appeared. The splitting products which we observed after digestion in the presence of 150 mM NaCl were, however, not significantly different from those observed in the presence

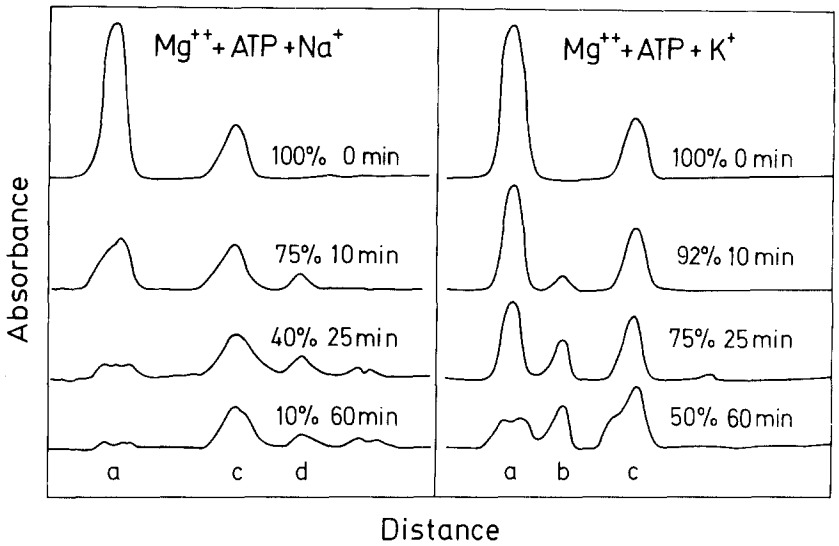


Fig. 4. Electrophoretograms of proteins after tryptic digestion of purified membrane-bound ($Na^+ - K^+$)-ATPase in the presence of 10 mM $MgCl_2$, 10 mM ATP plus 150 mM NaCl or in the presence of 10 mM $MgCl_2$, 10 mM ATP plus 150 mM KCl. Incubation with trypsin was performed as in Fig. 1. At different time intervals after the addition of trypsin, samples were mixed with trypsin inhibitor and assayed for ($Na^+ - K^+$)-ATPase activity or were prepared for SDS polyacrylamide gel electrophoresis as described under *Materials and Methods*. On each scan the time intervals and the remaining ($Na^+ - K^+$)-ATPase activity in percent of controls incubated without trypsin are given. The mol wt. of the peaks *a, b, c, d* are 120,000, 90,000, 60,000 and 42,000 daltons, respectively

of 150 mM KCl (digestion with a low trypsin concentration not shown, digestion with a high trypsin concentration, *see* Fig. 8*b*). This finding is in contrast to the data of Jørgensen [10] who described that different to the trypsin digestion in the presence of KCl the digestion in the presence of NaCl leads to the appearance of a large amount of 78,000-dalton splitting product which probably corresponds to the 75,000-dalton peptide observed by Lo and Titus [16] and to the 90,000-dalton peptide found by Giotta [5] and by our laboratory. Similar to our findings, Lo and Titus [16] observed in the presence of 100 mM NaCl only a small amount of 75,000-dalton peptide (about 10% of the amount which they found in the presence of 5 mM ATP). We found that the addition of Na^+ or K^+ led to different splitting products of the large polypeptide chain if Mg plus ATP were also present during the trypsin digestion. From Fig. 4 (low trypsin concentration) and from Fig. 8*c* (high trypsin concentration) it can be seen that a 90,000-dalton peptide was observed if the digestion was performed in the presence of Mg^{++}, ATP plus

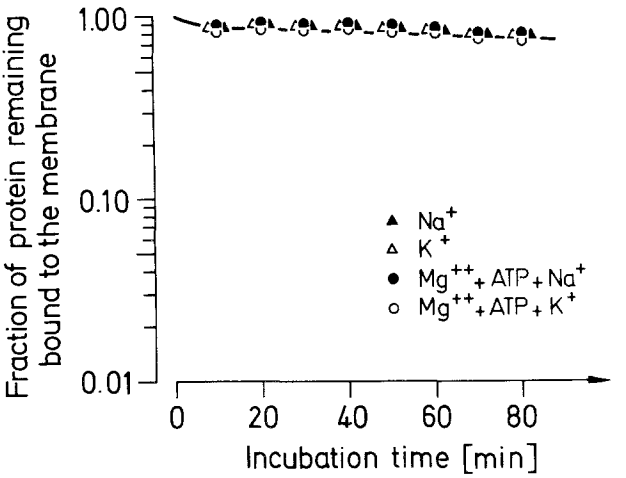


Fig. 5. Time courses of the release of $(Na^+ - K^+)$-ATPase protein from the $(Na^+ - K^+)$-ATPase membranes after trypsin digestion in the presence of different combinations of 10 mM Mg^{++}, 10 mM ATP, 150 mM Na^+ and 150 mM K^+. The trypsin digestion of the membrane-bound enzyme was performed as described in Fig. 1. At different time intervals samples were taken and trypsin inhibitor was added to a weight ratio of inhibitor to trypsin of 7.5:1. Then the $(Na^+ - K^+)$-ATPase membranes were washed twice by high speed centrifugation with 25 mM imidazole-HCl, pH 7.5, and the sediments were analyzed for their protein content

K^+ but was not detected if Mg^{++}, ATP plus Na^+ were present. In the presence of Mg^{++}, ATP plus Na^+ the large polypeptide was digested more quickly and a 42,000-dalton peptide was produced (Fig. 4, Fig. 8c)[3, 4].

The part of the $(Na^+ - K^+)$-ATPase protein which remained bound to the membrane after tryptic digestion was measured. Independent of whether Na^+ or K^+ or Mg^{++}, ATP plus Na^+ or Mg^{++}, ATP plus K^+ were present, only about the same small amount of protein was released into the supernatant (Fig. 5). Seventy-five percent of the $(Na^+ - K^+)$-ATPase protein remained membrane-bound after the enzyme was treated with trypsin for 80 min.

[3] Also in the presence of Mg^{++}, ATP plus K^+ sometimes a small amount of 42,000-dalton peptide was observed (*see* Fig. 8c).

[4] Studying the splitting of the large polypeptide chain in detail, we found that after treatment of $(Na^+ - K^+)$-ATPase with a low trypsin concentration (trypsin: 10 μg ml^{-1}, ratio of $(Na^+ - K^+)$-ATPase protein to trypsin: 67/1) a peptide with a molecular weight of 108,000 dalton appeared after 3 to 5 min tryptic digestion in the presence of Mg^{++}, ATP plus Na^+ or Mg^{++}, ATP plus K^+. A small amount of this peptide can also be observed after the digestion with higher trypsin concentrations (notice heterogeneity of band *a* after tryptic digestion in Fig. 4, *see* Fig. 8).

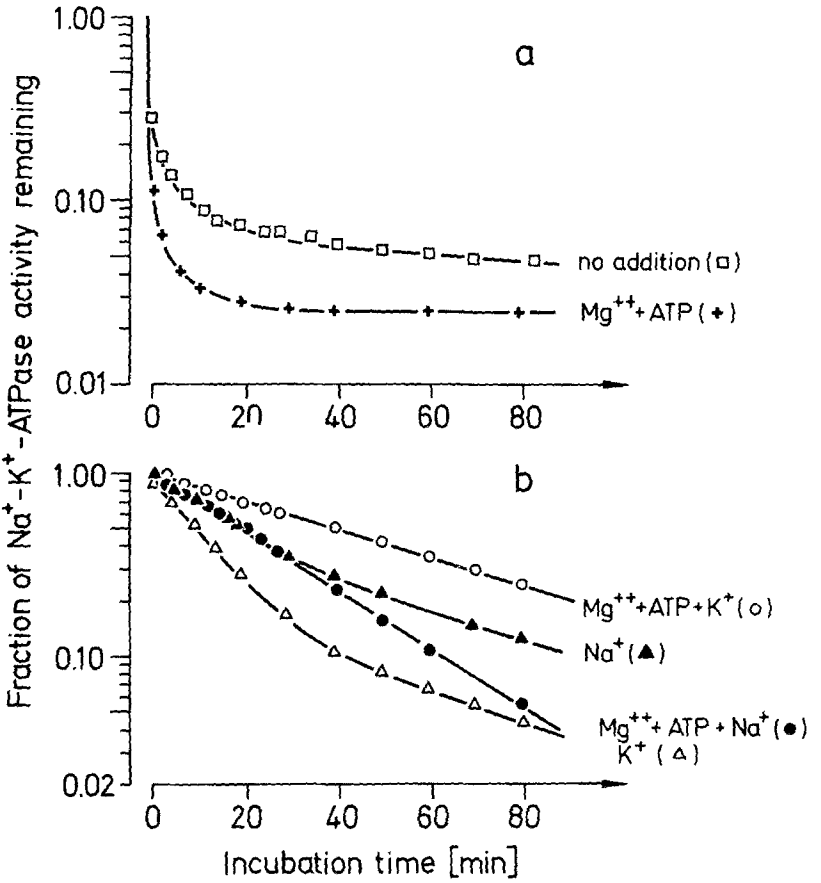


Fig. 6. Effect of Na^+ and K^+ on the trypsin digestion of a preparation of membrane-bound ($Na^+ - K^+$)-ATPase which contains tightly bound nucleotides. Different from the preparations of ($Na^+ - K^+$)-ATPase routinely employed, from which the nucleotides were removed by gel filtration in the presence of 50% (vol/vol) glycerol and 3 mM $MgCl_2$, in these experiments a ($Na^+ - K^+$)-ATPase preparation was used, which had been washed three times in the absence of $MgCl_2$ to remove the nucleotides. This preparation contained 0.8×10^{-9} moles ADP plus ATP per mg ($Na^+ - K^+$)-ATPase protein. Incubation with trypsin was performed as in Fig. 1. At different time intervals after the addition of trypsin the digestion was stopped by mixing with trypsin inhibitor, and samples were analyzed for ($Na^+ - K^+$)-ATPase activity (*a*, *b*) and for their content of 120,000 daltons protein (*c*). The combinations of 10 mM $MgCl_2$, 10 mM ATP, 150 mM NaCl and/or 150 mM KCl which were present during the trypsin digestion are indicated in the figure

Effect of Tightly Bound Nucleotides on the Trypsin Digestion

The effects of Na^+ and K^+ in the absence of ATP and Mg^{++} on the trypsin inactivation of the ($Na^+ - K^+$)-ATPase activity differ from those reported by Jørgensen [10, 11]. Jørgensen found that the membrane-

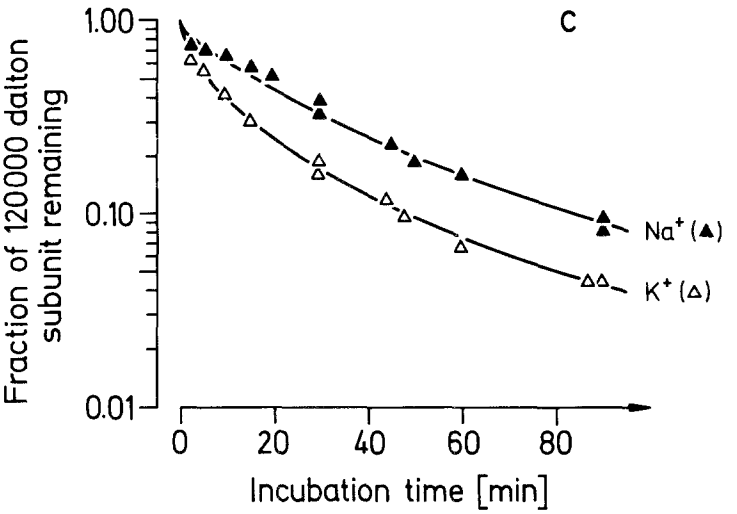


Fig. 6c

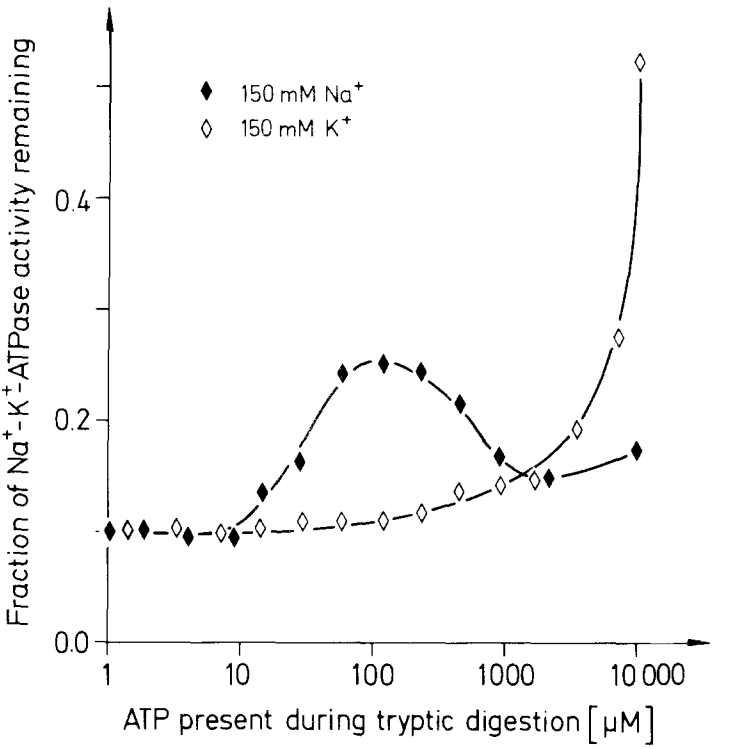


Fig. 7. Effect of Na^+ and K^+ on the ATP dependence of the trypsin inactivation of the membrane-bound ($Na^+ - K^+$)-ATPase. Purified ATP-free ($Na^+ - K^+$)-ATPase (0.1 mg protein per ml) was incubated at 37 °C for 40 min with trypsin (6 μg per ml) in a buffer containing varying concentrations of ATP, 25 mM imidazole-HCl, pH 7.5, and in addition 150 mM NaCl (◆) or 150 mM KCl (◇). The trypsin digestion was started and stopped and the ($Na^+ - K^+$)-ATPase activity was analyzed as in Fig. 1

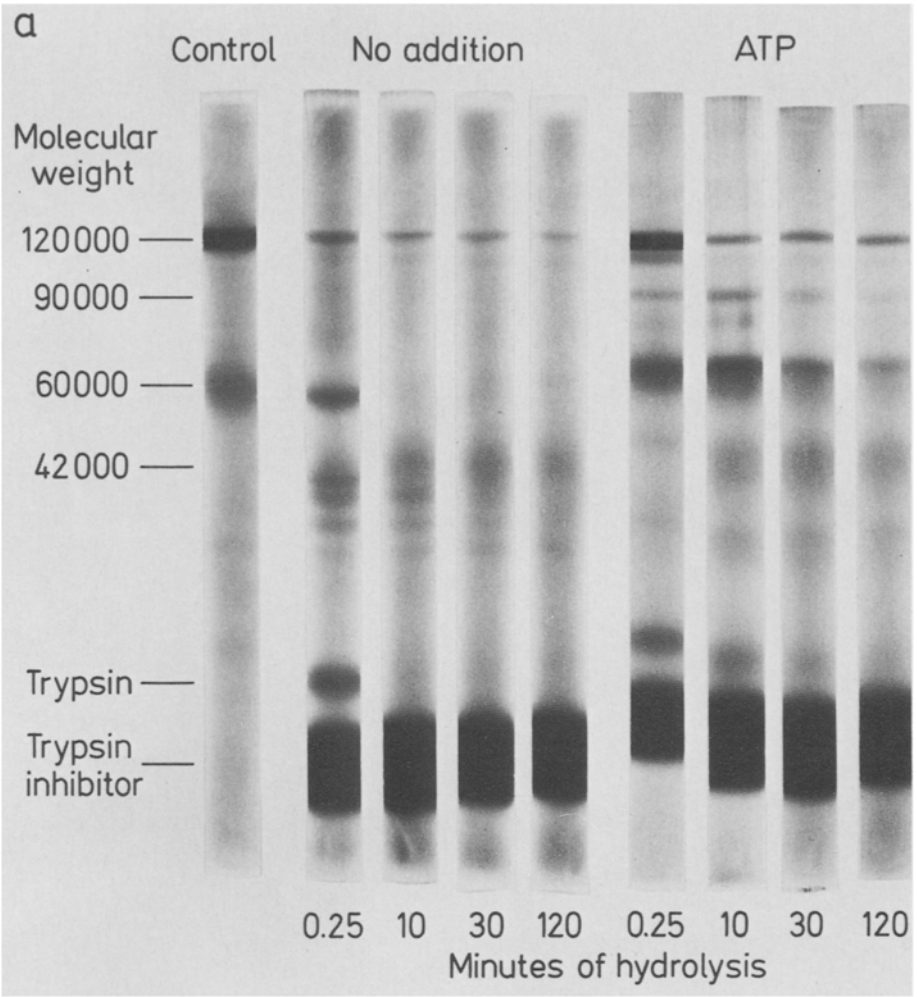
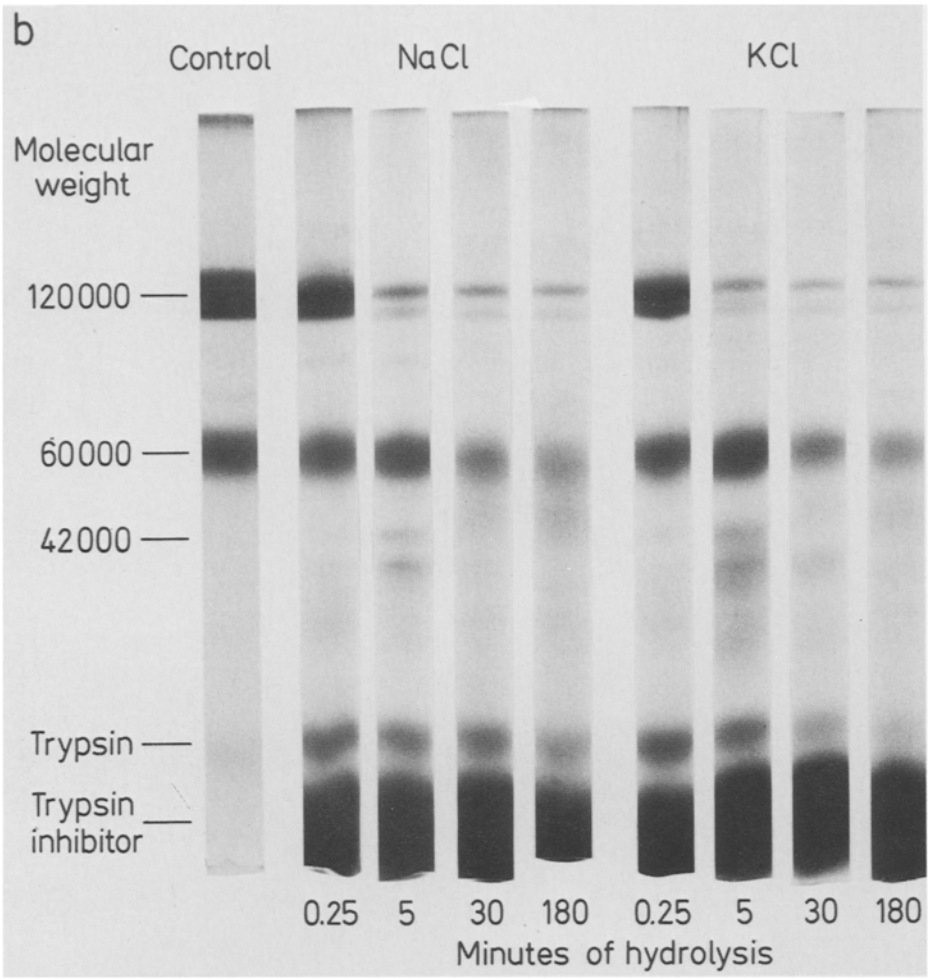


Fig. 8. Electrophoretic patterns of membrane-bound $(Na^+ - K^+)$-ATPase after digestion with a high trypsin concentration in the presence of different ATP and ligand combinations. Aliquots of purified membrane-bound $(Na^+ - K^+)$-ATPase (1 mg ml^{-1} protein) were incubated with trypsin at a concentration of 0.13 mg ml^{-1} in the presence of 20 mM imidazole HCl, pH 7.5, 2 mM EGTA, and the indicated combinations of 10 mM ATP, 10 mM $MgCl_2$, 150 mM NaCl, and 150 mM KCl. After the given time intervals, the digestion was stopped by adding trypsin inhibitor. Samples containing 15 µg of $(Na^+ - K^+)$-ATPase protein were prepared for polyacrylamide gel electrophoresis and were applied on top of each gel. Trypsin and the trypsin inhibitor appear as two bands in the low mol wt region

bound $(Na^+ - K^+)$-ATPase of the rabbit kidney was inactivated more slowly by trypsin in the presence of Na^+ than in the presence of K^+, that the time course of inactivation in the presence of K^+ was linear in a semilogarithmic plot while it was biphasic in the presence of Na^+, and that different splitting products appeared in the presence of Na^+



and K^+ (*see* above). Since Jørgensen [9, 10, 11] did not describe the method by which the nucleotides were removed from the $(Na^+ - K^+)$-ATPase preparation, we tested whether the presence of tightly bound nucleotides might explain the differences. Thus a series of trypsin digestion experiments was performed in which a membrane-bound $(Na^+ - K^+)$-ATPase preparation containing tightly bound nucleotides was used (Fig. 6). This preparation had been washed three times with 25 mM imidazole-HCl, pH 7.5, and contained 0.29 moles nucleotides (ATP plus ADP) per mole $(Na^+ - K^+)$-ATPase (assuming a mol wt of 360,000).

From Fig. 6*b* and *c* it can be seen that the results described by Jørgensen could be partially reproduced with this enzyme preparation. The trypsin inactivation of the $(Na^+ - K^+)$-ATPase activity proceeded more slowly in the presence of Na^+ than in the presence of K^+ and

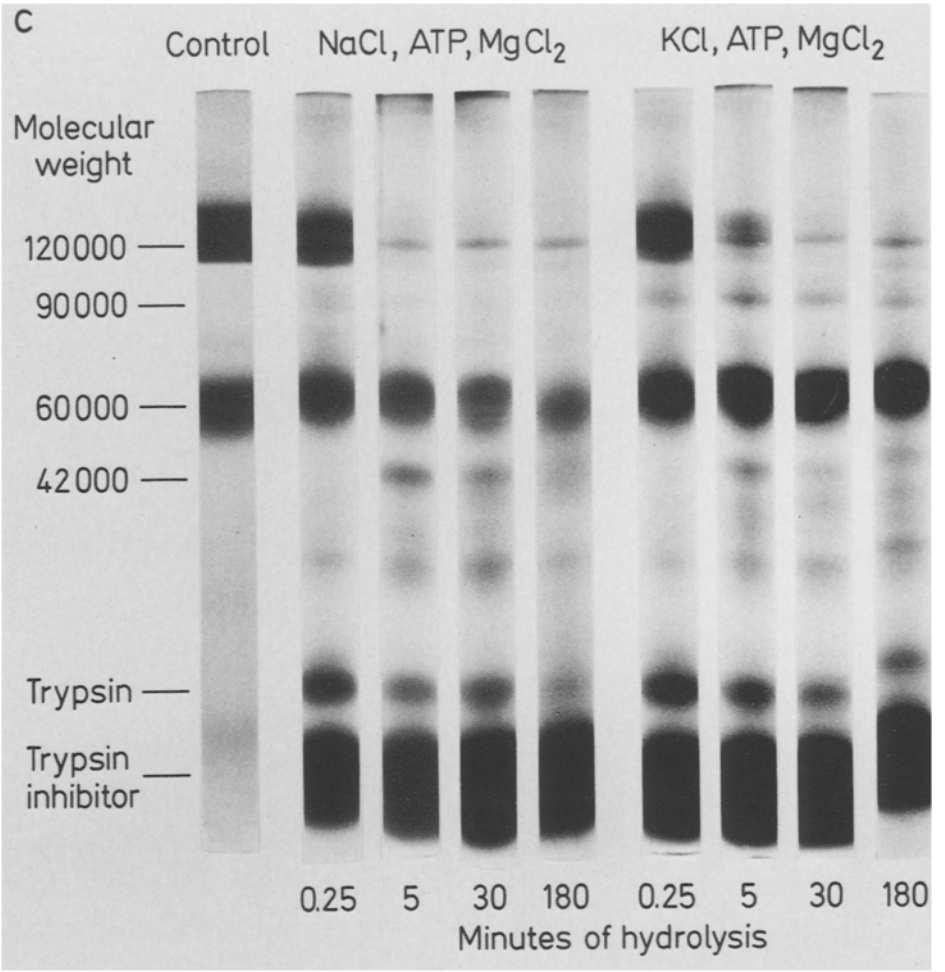


Fig. 8c

once more, the enzyme inactivation could be correlated to the degradation of the large chain and the degradation was slower with Na^+ than with K^+ (Fig. 6*c*). The monophasic inactivation curve in the presence of K^+ and the appearance of the 78,000-dalton peptide in the presence of Na^+ described by Jørgensen [10] were not found. If no ATP or cations were present, the trypsin inactivation of nucleotide-containing preparations was slower than that of the ATP-free membrane-bound enzyme (*compare* Fig. 6*a* with Fig. 1*a*). Comparing Fig. 1*a, b* and Fig. 6*a, b* it can also be seen that in the presence of 10 mM ATP plus various ligands the same inactivation curves were obtained whether or not the nucleotides had been completely removed before the test.

The ATP dependence of the trypsin inactivation of $(Na^+ - K^+)$-AT-

Pase activity in the presence of 150 mM NaCl or of 150 mM KCl was measured. Therefore ATP-free enzyme was incubated for 40 min with a low trypsin concentration in the presence of varying concentrations of ATP plus 150 mM NaCl or plus 150 mM KCl. From Fig. 7 it can be seen that in the presence of low concentrations of ATP (0.02–0.8 mM) the $(Na^+ - K^+)$-ATPase activity was protected by Na^+ but not by K^+. However, at high concentrations of ATP (3–10 mM) the presence of K^+ led to a much better protection of the $(Na^+ - K^+)$-ATPase activity than the presence of Na^+.

Degradation of the Small $(Na^+ - K^+)$-ATPase Subunit by Trypsin

Analyzing the time courses of trypsin degradation of the $(Na^+ - K^+)$-ATPase subunits it was found that the large polypeptide chain was much more susceptible to degradation by trypsin than the small chain. Thus, the large chain could be degraded to more than 95% and the $(Na^+ - K^+)$-ATPase activity was nearly completely inactivated while the small polypeptide chain was to about 95% intact. At this point about 75% of the enzyme protein remained bound to the membrane. Only after prolonged action of trypsin was the small chain digested. Incubating the ATP-free, membrane-bound $(Na^+ - K^+)$-ATPase at 37 °C with a high trypsin concentration (0.13 mg ml^{-1}), the large chain was digested nearly completely within 15 min and the $(Na^+ - K^+)$-ATPase activity was inactivated about 97%. The time courses of degradation of the small chain, 30 to 240 min after the addition of trypsin, were investigated in the presence of different ATP and ligand combinations. As can be seen from the gel pictures in Fig. 8 and from Fig. 9, the time course and degree of degradation of the small chain were highly dependent on the combinations of Na^+, K^+, Mg^{++} and ATP present during the trypsin digestion. ATP and ligands influenced the time course and degree of trypsin degradation qualitatively in the same way as they influenced the degradation of the large chain. Thus, at low ionic strength the highest rate of trypsin degradation of the small chain was obtained in the absence of ATP and ligands or in the presence of 10 mM $MgCl_2$ (Fig. 9*a*). Under these conditions about 93% of the small chain was digested after 2 hr. Analogous to the digestion of the large chain, the digestion of the small chain proceeded more slowly if ATP was present (Fig. 9*a*), so that after 2 hr 80% of this subunit was degraded. From Fig. 9*a* it can be seen further that this ATP effect was not observed if Mg^{++} was present at an equimolar concentration. At high ionic strength the rate of degrada-

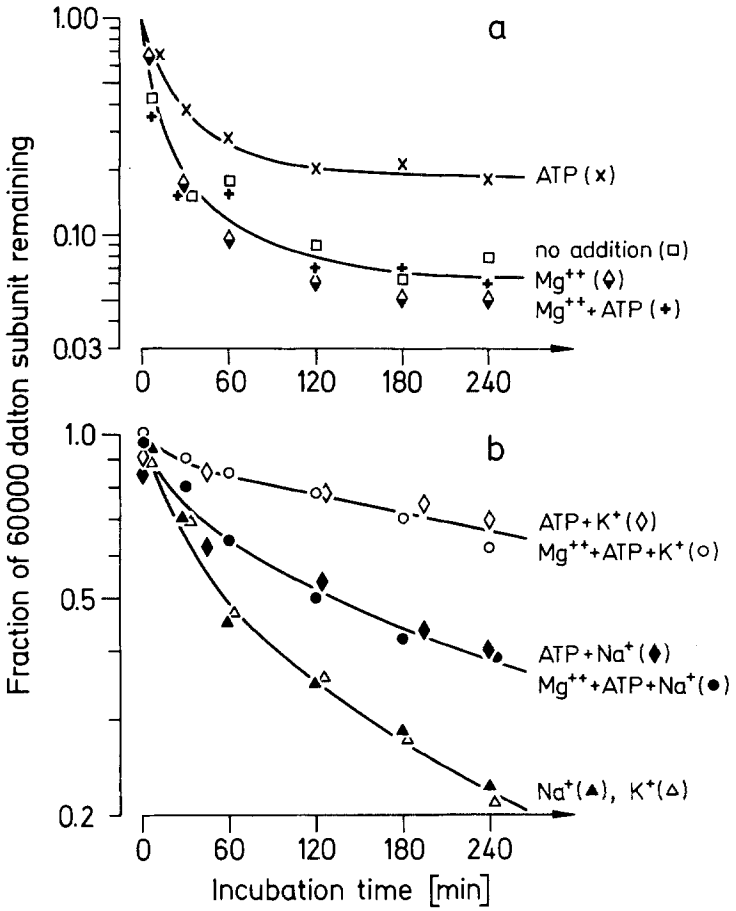


Fig. 9. Time courses of the degradation of the small polypeptide chain (60,000 dalton subunit of the membrane-bound ($Na^+ - K^+$)-ATPase) after digestion with a high trypsin concentration in the presence of different ATP and ligand combinations demonstrated in semilogarithmic plots. Incubations as in Fig. 8 with 1 mg ($Na^+ - K^+$)-ATPase protein and 0.13 mg trypsin in 1 ml buffer containing 20 mM imidazole-HCl, pH 7.5, 2 mM EGTA plus different combinations of 10 mM $MgCl_2$, 10 mM ATP, 150 mM NaCl and 150 mM KCl. In the figure mean values of three to six experiments are drawn

tion of the small chain was reduced. Na^+ and K^+, when present at 150 mM without ATP or Mg^{++}, did not differentially effect the degradation of the small chain (Fig. 9*b*). In the presence of 150 mM Na^+ or 150 mM K^+ about 65% of the small chain was degraded after 2 hr. If, however, ATP or Mg^{++} plus ATP were also present, Na^+ and K^+ influenced the trypsin degradation differentially. If 10 mM ATP plus 150 mM K^+ or if 10 mM ATP, 10 mM Mg^{++} plus 150 mM K^+ (Fig. 9*b*) were present, the small chain was protected from the trypsin degradation so that after 2 hr only about 20% of the small chain was digested.

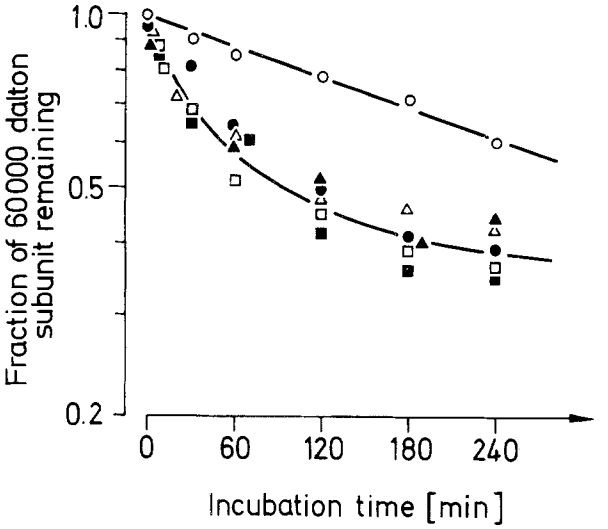


Fig. 10. Nucleotide specificity of the Na^+ and K^+ effects on the trypsin degradation of the small polypeptide chain of ($Na^+ - K^+$)-ATPase observed in the presence of Mg^{++} plus ATP. During the digestion, performed as in Fig. 8, 20 mM imidazole-HCl, pH 7.5, 2 mM EGTA, 10 mM $MgCl_2$ plus either 10 mM ATP (○●) or 10 mM adenylyl ($\beta-\gamma$-methylene) diphosphonate (△▲) or 10 mM ADP (□■) were present. During the action of trypsin 150 mM NaCl (closed symbols) or 150 mM KCl (open symbols) were present in addition

The small chain was digested more rapidly and to a higher extent if 10 mM ATP plus 150 mM Na^+ or if 10 mM ATP, 10 mM Mg^{++} plus 150 mM Na^+ (Fig. 9*b*) were present during the trypsin digestion, i.e., after 2 hr about 50% of the small chain was degraded.

To test whether the Na^+ and K^+ effect on the trypsin degradation in the presence of ATP and Mg^{++} was an ATP-specific phenomenon, ATP was replaced by ADP or by the ATP analog adenylyl ($\beta-\gamma$-methylene) diphosphonate (AMP-PCP). From Fig. 10 it can be seen that the time courses and degrees of trypsin degradation of the small chain were only different in the presence of Na^+ or K^+, if Mg^{++} and ATP were also present. In the presence of Mg^{++} plus ADP or of Mg^{++} plus AMP-PCP, Na^+ or K^+ did not differentially affect the degradation of the small chain.

Furthermore, it was tested whether in the presence of Mg^{++} plus ATP the effects of Na^+ and K^+ on the trypsin degradation of the small polypeptide chain are dependent on the integrity of the large chain or whether they can also be observed if the large chain has been degraded and the ($Na^+ - K^+$)-ATPase has been inactivated by trypsin beforehand. Thus membrane-bound ($Na^+ - K^+$)-ATPase (0.1 mg protein per ml) was incubated for 60 min at 37 °C with a low trypsin concentration (6 μg

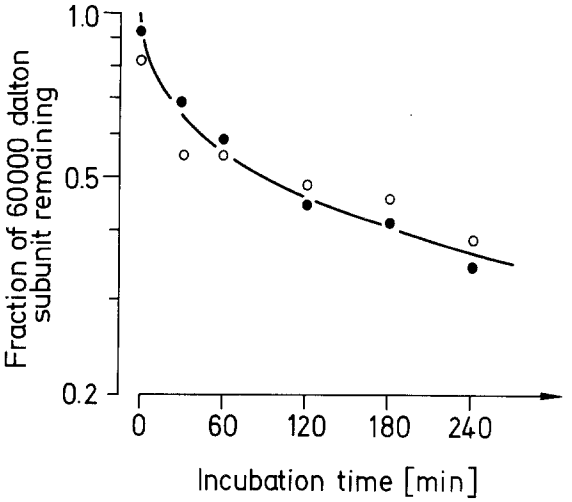


Fig. 11. Effects of Mg^{++}, ATP plus Na^+ and Mg^{++}, ATP plus K^+ on the trypsin degradation of the small polypeptide chain of a preparation of $(Na^+ - K^+)$-ATPase which had been inactivated by trypsin degradation of the large chain before. The membrane-bound enzyme was first incubated for 60 min at 37 °C with a low trypsin concentration (6 µg trypsin per ml, 0.1 mg $(Na^+ - K^+)$-ATPase protein per ml) in the presence of 25 mM imidazole-HCl, pH 7.5, and 150 mM choline chloride. After this procedure about 95% of the large polypeptide chain was degraded and the enzyme activity was decreased 97%. The $(Na^+ - K^+)$-ATPase membranes were spun down and digested with a high concentration of trypsin (experimental conditions as described in Fig. 8) in the presence of 10 mM $MgCl_2$, 10 mM ATP plus 150 mM NaCl (●) or plus 150 mM KCl (○)

ml^{-1}) in the presence of 150 mM choline chloride. After this procedure about 95% of the large chain was degraded and the enzyme activity was decreased 97%, whereas the small polypeptide chain remained intact. In a second step the $(Na^+ - K^+)$-ATPase membranes were concentrated by centrifugation and then treated with a high trypsin concentration (0.13 mg ml^{-1}) in the presence of 10 mM ATP, 10 mM $MgCl_2$ plus 150 mM NaCl or plus 150 mM KCl. From Fig. 11 it can be seen that the degradation of the small polypeptide chain was not affected by Na^+ or K^+ in the presence of Mg^{++} plus ATP, if the $(Na^+ - K^+)$-ATPase had been inactivated by trypsin digestion of the large polypeptide chain beforehand.

Discussion

Tryptic digestion of proteins, which are to a significant portion embedded in the membrane, is an appropriate method to study their structural changes, if only few L-lysyl and L-arginyl bonds of the proteins are accessible to the trypsin hydrolysis and if their accessibility to trypsin is altered during their structural changes. Several authors [5, 10, 11,

16] have demonstrated that the tryptic digestion of the purified ($Na^+ - K^+$)-ATPase is rather specific and that the addition of the ligands Na^+, K^+, Mg^{++} and ATP, which have no specific effects on the activity and stability of trypsin [10], lead to an alteration of the sites of trypsin attack [5, 10, 11, 16] and to an alteration of the time course of trypsin inactivation of the ($Na^+ - K^+$)-ATPase activity [5, 10]. We could extend the previous findings by measuring the trypsin inactivation of the ($Na^+ - K^+$)-ATPase activity as well as the digestion of the large and the small polypeptide chains of the ($Na^+ - K^+$)-ATPase and the corresponding release of ($Na^+ - K^+$)-ATPase protein from the membrane in the presence of different trypsin concentrations and of various ligand combinations. Moreover, the above reported trypsin digestion experiments on the membrane-bound ($Na^+ - K^+$)-ATPase help to further the understanding of the topological localization of the ($Na^+ - K^+$)-ATPase subunits within the cell membrane.

Characteristics of ATP and Ligand-Induced Structural Changes of the ($Na^+ - K^+$)-ATPase

In agreement with previous reports [1, 5, 10] it was found that primarily the high molecular weight subunit was attacked by trypsin and that the ($Na^+ - K^+$)-ATPase activity decreased in parallel to the degradation of this subunit. The low molecular weight subunit of the ($Na^+ - K^+$)-ATPase was still intact after the complete degradation of the large polypeptide chain. After a long incubation time with a high trypsin concentration the small polypeptide chain was also degraded. As presented here and also previously reported from our laboratory [12], ATP and ligands influence not only the rate of trypsin inactivation of the ($Na^+ - K^+$)-ATPase activity and the degradation of the large polypeptide chain but also the rate of degradation of the small chain of the ($Na^+ - K^+$)-ATPase. Measuring the effects of different ATP and ligand combinations on the trypsin digestion it was found that the ($Na^+ - K^+$)-ATPase activity, the degradation of the large chain as well as the degradation of the small chain were affected in parallel. Thus, certain ATP and ligand combinations did alter neither the degree of degradation of the large polypeptide chain nor the degree of degradation of the small chain, whereas with other combinations of ATP and ligands the degree of degradation of both ($Na^+ - K^+$)-ATPase subunits was equally reduced or increased. This finding means that binding of ATP and certain ligand combinations

regulates the synchronous exposure of both polypeptide chains. If in addition to this joint movement of the large and the small polypeptide chain the position of the $(Na^+ - K^+)$-ATPase subunits to each other is altered, this must take place in a way that both subunits are exposed or protected in parallel. Since the protein fragments were only partially released from the membrane during tryptic digestion, a shielding of the small by the large polypeptide chain may occur. It cannot be excluded that ligand-induced structural states of the large chain may survive the trypsin proteolysis and may lead to a differential shielding of the small polypeptide chain. However, the observed parallelism of the effects of ATP and ligands on the degradation of both $(Na^+ - K^+)$-ATPase subunits suggests that different degrees of shielding of the small subunit by the large one are not the main reason for the observed digestion patterns.

The finding that the $(Na^+ - K^+)$-ATPase activity decreased in parallel to the degradation of the large polypeptide chain, which has also been found by Jørgensen [10], and by Giotta [5], the latter working with the solubilized enzyme, may be taken as evidence that the hydrolytic function of the $(Na^+ - K^+)$-ATPase is localized in the high molecular weight subunit of the enzyme. This concept is further validated by the findings (i) that the large chain is phosphorylated in the presence of Na^+, Mg^{++} and ATP and dephosphorylated when K^+ is added [2], (ii) that the cardiac glycoside binding sites are on the large chain [22], and (iii) that a preparation of $(Na^+ - K^+)$-ATPase from pig brain was obtained which consisted only of a large subunit and possessed a high specific Na^+, K^+ and Mg^{++} stimulated ATPase activity [20]. Thus, it can be assumed that the binding sites for ATP, Na^+, K^+ and Mg^{++} are located on the high molecular weight subunit. As far as ATP binding is concerned this could be directly shown by radioactive labeling the $(Na^+ - K^+)$-ATPase with the covalently binding ATP analogue NbS^6ITP[5] (F. Hulla & H. Koepsell, *unpublished*). Hence, as ATP and most probably also the ligands bind at the large polypeptide chain, it must be assumed that ATP and/or ligands primarily alter the structure of the large chain. The structural change of the large subunit is supposed to lead to a synchronous movement of the small subunit, which by crosslinking experiments has been demonstrated to be closely associated to the large one [15]. The described finding that the differential effects

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[5] 6-[(3-carboxy-4-nitrophenyl)thiol]-9-βD-ribofuranosylpurine 5′-triphosphate which had been labeled with ^{32}P in β- and γ-positions.

of Na^+ plus ATP and K^+ plus ATP on the trypsin digestion of the small polypeptide chain were only observed if the large chain was intact, when ATP and ligands were added to the enzyme, supports this concept. Moreover, the structural state of the small subunit, which has been modified by ligand-induced structural alterations of the large subunit, remained unchanged when the large chain had been degraded. This can be derived from the observation that, in experiments where first ATP and ligands and then trypsin was added to the enzyme, the speed of degradation of the small subunit was still dependent on ATP and ligands when the large subunit had already been completely degraded.

By the proposed concept that ATP- and ligand-induced structural changes of the large polypeptide chain lead to a synchronous movement of the small chain, the previously described finding that antibodies directed against the small chain inhibit the $(Na^+ - K^+)$-ATPase activity [7, 8, 21], can be explained. Thus, by blocking the movement of the small polypeptide chain, structural changes of the large chain which are linked to the ATP hydrolysis may be prevented, provided the coupling between the two polypeptide chains is intact. Whether this coupling can actually be destroyed without an effect on the hydrolytic activity, as the results of Nakao and coworkers [20] suggest, and whether the coupling between the $(Na^+ - K^+)$-ATPase subunits is necessary for Na^+ and K^+ transport has to be elucidated in the future.

Dependence of the $(Na^+ - K^+)$-ATPase Structure on ATP and Ligands

The trypsin digestion experiments demonstrated that different structural states of the $(Na^+ - K^+)$-ATPase molecule can be distinguished, which we have also shown by antibody inhibition studies [14]. Thus with both methods different enzyme conformations in the presence of Mg^{++}, ATP plus Na^+ and in the presence of Mg^{++}, ATP plus K^+ were detected, whereas no structural alterations of the enzyme specific to the addition of Na^+ or K^+ could be found in the absence of ATP. As it is improbable that all possible structural states of the $(Na^+ - K^+)$-ATPase can be distinguished by antibody inhibition studies or by trypsin digestion experiments, different results obtained by the two methods are not contradictory but must be considered to be supplementary. Thus, an effect of Mg^{++} on the structure of the $(Na^+ - K^+)$-ATPase in the absence of ATP was not observed in the trypsin digestion experiments,

although it has been detected by antibody inhibition and by several other methods (*review* Ref. 14). On the other hand, it was found similar to the results of Giotta, who performed trypsin digestion experiments with a solubilized ($Na^+ - K^+$)-ATPase preparation [5], that in the absence of Mg^{++} binding of ATP leads to a protection of the enzyme which is considered to be due to a structural change of the protein, whereas binding of ATP has no effect on the degree of antibody inhibition of ($Na^+ - K^+$)-ATPase activity [14]. Different from the antibody inhibition experiments, in which different degrees of antibody inhibition were obtained in the presence of Na^+ or K^+ provided ATP plus Mg^{++} were present [14], Na^+ and K^+ also showed different effects on the trypsin action if ATP but no Mg^{++} was present. Thus, it can be concluded that Na^+ and K^+ modulate the ($Na^+ - K^+$)-ATPase structure if ATP has bound – but in a different, probably more extensive manner – if ATP plus Mg^{++} have bound to the enzyme. It was demonstrated that the differential effect of Na^+ and K^+ on the trypsin digestion in the presence of ATP is highly dependent on the ATP concentration. Thus, in the presence of low ATP concentrations (e.g., 0.1 mM) the enzyme is protected much more by Na^+ than by K^+, whereas in the presence of high concentrations of ATP (e.g., 10 mM) the enzyme is strongly protected by K^+ and to a much smaller degree by Na^+. If the nucleotides ATP and ADP which were present during the preparation of the enzyme were not completely removed, Na^+ and K^+ showed differential effects on the trypsin digestion if no further ATP was added. These effects were similar to those observed when low ATP concentrations were added to the ATP-free enzyme. The results obtained with enzyme containing tightly bound nucleotides are similar to those reported by Jørgensen [10]. The fact that Jørgensen's results were not completely reproduced is probably due to a different amount of tightly bound nucleotides in his and our enzyme preparations. Thus by trypsin digestion experiments as well as by antibody inhibition studies [14] no indication was obtained that, in the absence of ATP, Na^+ and K^+ alter the ($Na^+ - K^+$)-ATPase structure differentially. From the trypsin digestion experiments it can be concluded that the ($Na^+ - K^+$)-ATPase structure is altered by binding of Na^+ in a different way than by binding of K^+, provided ATP has also bound to the enzyme. The data further suggest that the effects of Na^+ and K^+ on the enzyme structure are different when one or more nucleotides have bound to the ($Na^+ - K^+$)-ATPase molecule. This interpretation fits into the concept that the functioning ($Na^+ - K^+$)-ATPase complex has more than one ATP binding site, which has been hypothesized from kinetic data

demonstrating a negative cooperativity for the ATP binding [13, 23] as well as the ATP dependence of the $(Na^+ - K^+)$-ATPase activity [3, 13, 23].[6]

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[6] The number of ATP binding sites in the functioning $(Na^+ - K^+)$-ATPase complex has not been determined unambiguously. Measuring the ATP binding to the $(Na^+ - K^+)$-ATPase, we could distinguish more than two different apparent dissociation constants (*unpublished*). Recently Grosse and coworkers [6] could fit experimental data on the effect of ATP, K^+ and/or Na^+ on the rate of inactivation of $(Na^+ - K^+)$-ATPase activity of the pig kidney enzyme by the SH-group reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to a model assuming two coexisting ATP binding sites. For the E-ATP complex in the absence of Na^+ and K^+ they calculated an apparent dissociation constant of 5 μM and for the $E\text{-}(ATP)_2$ complex a 48-times higher value. The $K_{0.5}$ value for the high affinity binding to the rat kidney $(Na^+ - K^+)$-ATPase has been determined to be in the absence of Na^+ and K^+ 0.55 μM [13]. The half-maximal concentration for the above-described ATP effect on the trypsin digestion of the $(Na^+ - K^+)$-ATPase is 0.028 mM, that is 51 times higher than the $K_{0.5}$ value for the high-affinity ATP binding.

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