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PURIFICATION AND CHARACTERIZATION OF (Na^+, K^+)-ATPase

V. CONFORMATIONAL CHANGES IN THE ENZYME. TRANSITIONS BETWEEN THE Na-FORM AND THE K-FORM STUDIED WITH TRYPTIC DIGESTION AS A TOOL

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SUMMARY

1. Purified (Na^+, K^+)-ATPase consisting of membrane fragments was digested with trypsin. The time course of enzyme inactivation was related to the electrophoretic pattern of native and cleaved proteins remaining in the membrane.

2. Differences in both the inactivation kinetics and the cleavage of the large chain (mol. wt 98 000) allow distinction of two patterns of tryptic digestion of (Na^+, K^+)-ATPase seen with Na^+ or K^+ in the medium.

3. With K^+, the inactivation of (Na^+, K^+)-ATPase is linear with time in semilogarithmic plots and the activity is lost in parallel with cleavage of the large chain to fragments with molecular weights 58 000 and 48 000.

4. With Na^+, the inactivation curves are biphasic. In the initial phase of rapid inactivation, 50 % of the activity is lost with minor changes in the composition of the large chain. In the final phase, the large chain is cleaved at a low rate to a fragment with a molecular weight of 78 000.

5. It is concluded that the regions of the large chain exposed in the presence of K^+ are distinct from the regions exposed in presence of Na^+ and that two conformations of (Na^+, K^+)-ATPase can be sensed with trypsin, a (t)K-form and a (t)Na-form.

6. Reaction of the (t)K-form with ATP cause transition to the (t)Na-form. Relatively high concentrations of ATP are required and Mg^{2+} is not necessary. Phosphorylation of (Na^+, K^+)-ATPase is accompanied by transition from the (t)Na-form to the (t)K-form. Previous kinetic data suggest that these conformational changes are accompanied by shifts in the affinities of the enzyme for Na^+ and K^+.

INTRODUCTION

Digestion with trypsin (EC 3.4.21.4) combined with sodium dodecyl sulfate gel electrophoresis has provided valuable information about the disposition of

proteins in membranes [1] and about the proteins of specific membrane components such as the Ca^{2+}-ATPase of the sarcoplasmic reticulum [2, 3].

Treatment with trypsin readily inactivates (Na^+, K^+)-ATPase [4–6], but reports on the effects of tryptic digestion on the protein composition of the enzyme are not available.

The purpose of this work has been to study the consequences of trypsin treatment of purified (Na^+, K^+)-ATPase [7, 8] consisting of membrane fragments with a distinct substructure of 40–50 Å particles [9]. This preparation contains only two protein components and the large catalytic subunit of (Na^+, K^+)-ATPase forms 60–70 % of the total protein [10, 11]. It was therefore possible to relate the loss of (Na^+, K^+)-ATPase activity with trypsin to the content of this protein in the membranes and to identify the cleaved fragments arising by tryptic digestion.

Differences in the substructure of proteins [12] and conformational changes of enzyme proteins [13] are reflected in the kinetics of tryptic digestion. The intact membrane structure forms a barrier to proteolysis [14] and recent reports show that changes in structure or location of membrane proteins [15] and their interaction with lipids [16] may alter the pattern of cleaved fragments after tryptic digestion. Kinetic data suggest that (Na^+, K^+)-ATPase undergoes conformational changes on reaction with Na^+, K^+, ATP and Mg^{2+} (see ref. 17). It was therefore examined if these ligands can alter the electrophoretic pattern of cleaved fragments and the kinetics of tryptic inactivation of the purified (Na^+, K^+)-ATPase.

EXPERIMENTAL

(Na^+, K^+)-ATPase was purified as described recently [7, 8] from the outer medulla of rabbit kidney by incubation of a microsomal fraction with sodium dodecyl sulfate and ATP followed by an isopycnic-zonal centrifugation.

Treatment with trypsin from bovine pancreas (Sigma, twice crystallized, salt free) was carried out at 37 °C in 25 mM imidazole buffer, pH 7.5, with weight ratios of trypsin to (Na^+, K^+)-ATPase protein varying from 1 : 40 to 1 : 10. The cations were added as their chloride salts. In experiments with ATP, ADP and Mg^{2+}, the ionic strength was kept constant by exchanging ATP or ADP with Tris-chloride and $MgCl_2$ with choline chloride. Digestion was stopped by mixing with Soyabean trypsin inhibitor (Sigma, type I-S) to a weight ratio of inhibitor to trypsin of 2–3 : 1 and aliquots were taken out for assay of (Na^+, K^+)-ATPase as before [7]. In studies of the time course of inactivation of (Na^+, K^+)-ATPase by trypsin the activities were calculated as fractions of original activities remaining and were plotted against time in semilogarithmic plots. Zero time activities were determined before the addition of trypsin. For analysis of the protein composition, the samples were washed twice by high-speed centrifugation and aliquots of the sediments were taken out for assay of (Na^+, K^+)-ATPase, for analysis of protein by the method of Lowry et al. [18] as described recently [7], and for preparation of samples for polyacrylamide gel electrophoresis in sodium dodecyl sulfate. This was done as before [7] in 12-cm separating gels with 1.5-cm stacking gels [19]. 15–20 μg protein was applied per gel and electrophoresis was performed at 20 °C and a current of 2–3 mA per gel for 15–20 h. The gels were stained with Coomasie brilliant blue [19]. The molecular weights of the polypeptide chains were determined by calibration against known standards: β-

galactosidase (mol. wt 130 000), phosphorylase a (mol. wt 94 000), pyruvatekinase (mol. wt 57 000), lactate dehydrogenase (mol. wt 36 000) and cytochrome *c* (mol. wt 11 700) [19].

The proteolytic activity of trypsin was determined by the casein digestion method [20] using nitrated casein (Calbiochem). The undegraded casein was precipitated with 5 % trichloroacetic acid and centrifugation. The supernatant was made alkaline with 2 M Tris and absorbance was read at 428 nm to avoid interference by ADP or ATP. The esterase activity of trypsin toward *p*-toluenesulfonyl-L-arginine methyl ester (Calbiochem) was determined by the spectrophotometric procedure [21].

RESULTS

The purified preparations of (Na^+, K^+)-ATPase had specific activities of 32–40 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein and no ouabain-insensitive Mg^{2+}-ATPase activity. The chemical and enzymatic properties of this preparation and estimates of its purity with respect to protein and concentration of active sites were reported recently [7, 10, 11]. The preparation consisted of flat or cup-shaped membrane fragments, 1000–3000 Å in diameter, with a distinct substructure of 40–50 Å particles arranged in clusters between smooth areas [9, 10].

A controlled and relatively slow rate of inactivation of (Na^+, K^+)-ATPase by trypsin was obtained by adjusting the ratio of trypsin to enzyme protein. In agreement with Somogyi's results [5] it was found that salts counteract the tryptic inactivation of (Na^+, K^+)-ATPase. It was therefore important to keep the ionic strength constant in examination of the effects of ions and nucleotides on the digestion. Sucrose protects against the inactivation of Ca^{2+}-ATPase in sarcoplasmic reticulum vesicles [2], but it had only a negligible effect on the inactivation of (Na^+, K)-ATPase. The inactivation of (Na^+, K^+)-ATPase by trypsin was stopped completely by addition of trypsin inhibitor if the weight ratio of inhibitor to trypsin was 1 or higher. Incubation of the purified preparation for 1 h at 37 °C without trypsin or with trypsin inhibitor added prior to addition of trypsin did not alter the (Na^+, K^+)-ATPase activity.

Effects of NaCl and KCl on the digestion of (Na^+, K^+)-ATPase by trypsin

The semilogarithmic plots in Fig. 1 show that tryptic inactivation of (Na^+, K^+)-ATPase with K^+ in the medium was linear with time, indicating that the process is pseudo first order in enzyme concentration. With Na^+, the curves require fitting by two exponentials. The rate constants for inactivation in the faster initial phase was more than one order of magnitude higher than the rate constants for the slower phase and both constants were increased with an increase in the concentration of trypsin. The fraction of the activity lost in each of the two phases was close to 0.5 and this distribution was independent of the trypsin concentration.

The purpose of the experiment shown in Fig. 2 was to examine the effect of Na^+ and K^+ on the inactivation in the final phase of digestion. It is seen that the curves had a sigmoid shape and that relatively high concentrations of Na^+ were required to decrease the rate of inactivation. The apparent Na^+/K^+ affinity ratio for half maximum effect was 0.33 : 1 and similar to the affinity ratio found for the sodium site of (Na^+, K^+)-ATPase in the absence of ATP [22, 23].

Examination of the cation specificity (Fig. 3) showed that linear inactivation

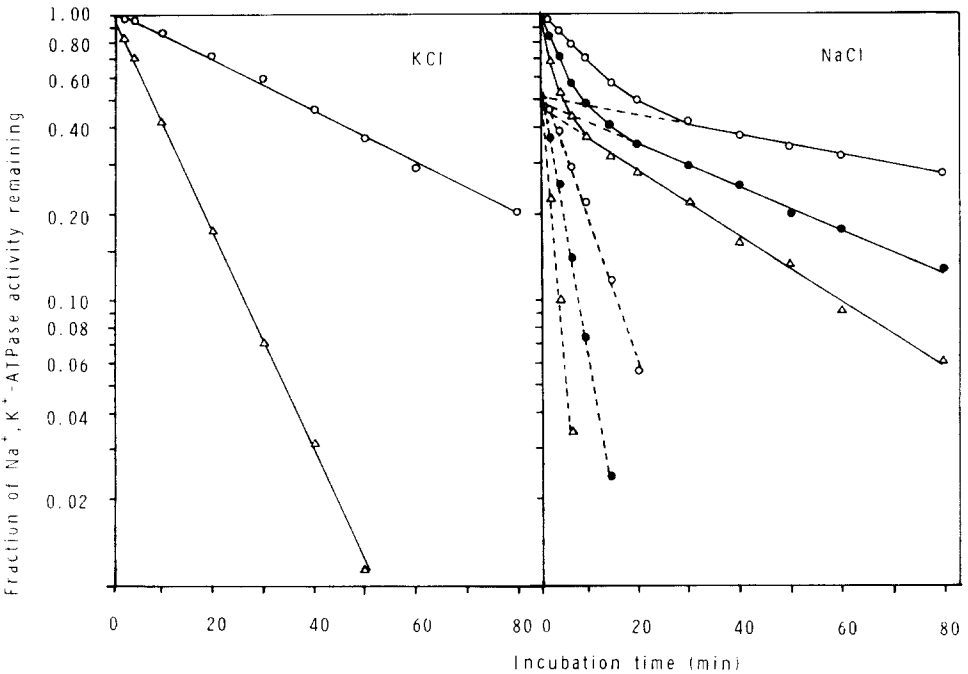

Fig. 1. Semilogarithmic plots of inactivation of (Na^+, K^+)-ATPase by trypsin in presence of KCl and NaCl. Aliquots of the (Na^+, K^+)-ATPase preparation containing 100 μg protein were incubated at 37 °C in 1 ml with 25 mM imidazole, pH 7.5 (37 °C), 150 mM KCl or 150 mM NaCl and 3 (○), 6 (●), and 10 μg (△) trypsin. Digestion was started by addition of trypsin. At the indicated intervals 50 μl of the incubation medium was mixed with 20 μl 25 mM imidazole, pH 7.5, containing 1 μg trypsin inhibitor. After completion of the incubation 25 μl of this mixture was transferred to test tubes containing 3 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl, 3 mM ATP (Tris salt), 30 mM histidine, pH 7.5 (37 °C) and where appropriate 1 mM ouabain in a total volume of 1 ml. After 5 min at 37 °C the reaction was stopped with 100 μl 50 % trichloroacetic acid and P_i was measured [27]. The (Na^+, K^+)-ATPase activity was taken to be the difference in activity with and without ouabain.

The rate constants for the curves obtained with KCl were 0.020 (○), and 0.091 (△). The final slopes of the curves obtained with NaCl were extrapolated to zero time. The values on the dotted lines were obtained by subtraction of the values along the extrapolated slope from the observed values [28]. The rate constants calculated from the slopes of the dotted lines were 0.116 (○), 0.231 (●), and 0.471 (△). The rate constants calculated from the final slopes were 0.0080 (○), 0.018 (●), and 0.027 (△).

curves were obtained with the congeners of potassium, Rb^+ and Cs^+. The curve seen with Li^+ occupied an intermediate position and the biphasic curves obtained with choline and Tris in the medium were similar to the curve obtained with Na^+ (Fig. 1). This result agrees with the known specificity of (Na^+, K^+)-ATPase for cations. Rb^+ and Cs^+ can replace K^+ as activator of ATP hydrolysis and Li^+ may act both as Na^+ and K^+ [25]. Choline [24] and Tris [26] have sodium-like effects on partial reactions of the enzyme but with a much lower affinity than that found for Na^+.

Fig. 4 shows that extensive digestion with trypsin was required to release significant amounts of protein from the membrane-bound (Na^+, K^+)-ATPase. It is seen that 90–95 % of the protein remained joined with the membranes after tryptic

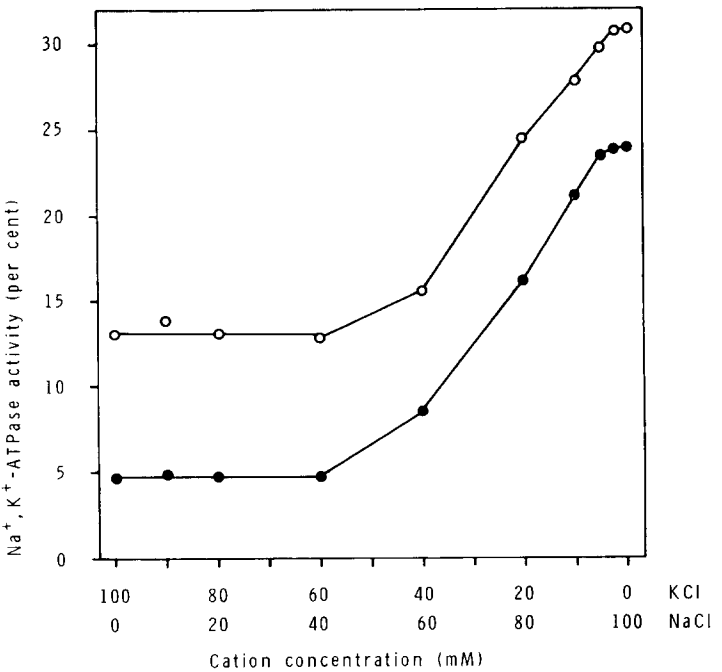

Fig. 2. Effect of Na^+ + K^+ on the (Na^+, K^+)-ATPase activity remaining after digestion with trypsin for 20 min (○) and 30 min (●). Incubation as described in Fig. 1, but with 50 μg (Na^+, K^+)-ATPase protein in 0.5 ml with NaCl and KCl in the concentrations shown on the abcissa and 4 μg trypsin. Mixing with trypsin inhibitor and assay of (Na^+, K^+)-ATPase as in Fig. 1.

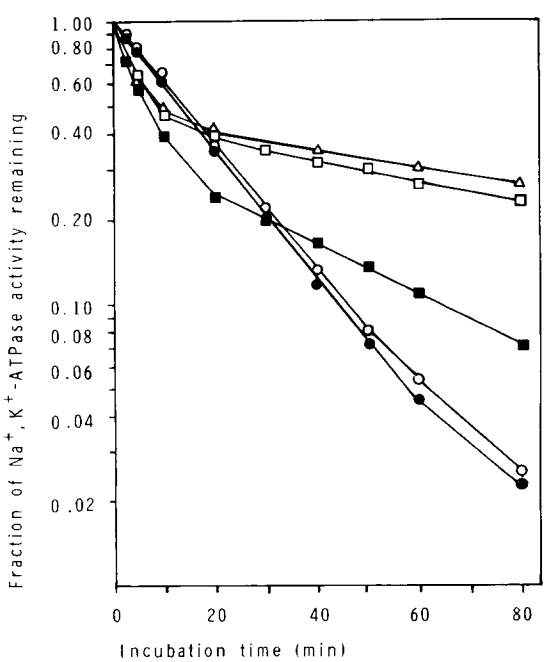

Fig. 3. First-order plots of inactivation of (Na^+, K^+)-ATPase by trypsin in presence of RbCl (●), CsCl (○), LiCl (■), choline chloride (□), and Tris-chloride (△). Incubation as in Fig. 1 with 100 μg (Na^+, K^+)-ATPase protein and 8 μg trypsin in 1.0 ml and 150 mM salt. Mixing with trypsin inhibitor and enzyme assay as in Fig. 1.

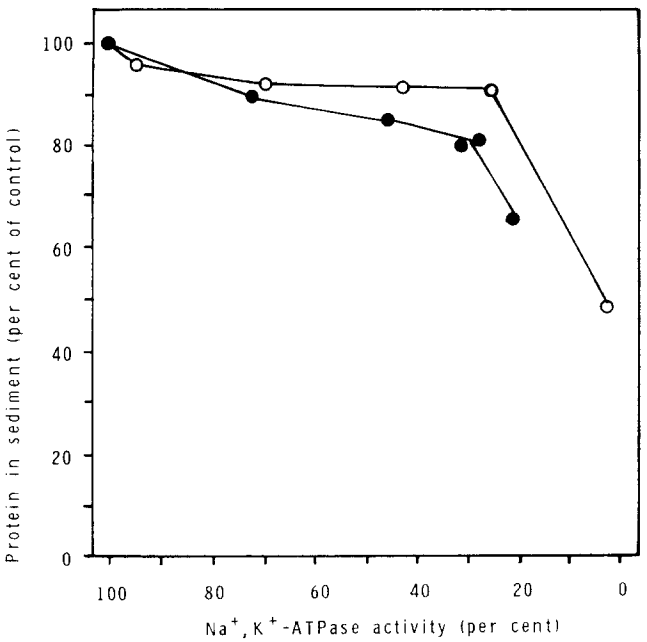

Fig. 4. Relationship between the fraction of protein remaining in the membranes and the activity of (Na^+, K^+)-ATPase after tryptic digestion in presence of KCl (○) and NaCl (●). Aliquots of the (Na^+, K^+)-ATPase preparation containing 150 μg protein were incubated at 37 °C in 1 ml with 25 mM imidazole, pH 7.5, 2–8 μg trypsin and 100 mM KCl or 100 mM NaCl for 5–40 min. Digestion was stopped by mixing with 1 ml 25 mM imidazole, pH 7.5, containing 20 μg trypsin inhibitor. The mixtures were centrifuged for 90 min at 40 000 rev./min in the Ti-60 Beckman rotor. The sediments were washed again with 2 ml 25 mM imidazole, pH 7.5, containing 20 μg trypsin inhibitor and were resuspended in 0.15 ml 25 mM imidazole, pH 7.5. Assay for (Na^+, K^+)-ATPase as described in Fig. 1. The results are expressed in percent of controls incubated without trypsin.

digestion in presence of K^+ resulting in loss of up to 75 % of the activity. Digestion with Na^+ in the medium released slightly more protein from the membranes than did digestion with K^+ to the same levels of activity. The proteins in the supernatant fluids were concentrated by lyophilization and analyzed by gel electrophoresis in sodium dodecyl sulfate. Apart from bands of trypsin and trypsin inhibitor only weak, diffuse bands of peptides migrating with the marker were visible (not shown). The peptides released from the membranes were thus too small for characterization by this technique.

The purified (Na^+, K^+)-ATPase contained two protein components [7, 11], a large chain and a smaller chain with apparent mol. wts of 98 000 and 58 000. The electrophoretograms of the protein remaining in the membranes (Fig. 5) shows that the pattern of tryptic cleavage with K^+ in the medium was distinctly different from that seen with Na^+. With K^+ in the medium, the large chain was initially cleaved to two fragments; fragment F_c (Fig. 5, peak c) with an apparent molecular weight of 58000 and a smaller fragment of 48000 appearing as a shoulder on peak c. Fragment F_d (Fig. 5, peak d) with a molecular weight of 42 000 appeared when most of the large chain had been cleaved suggesting that it was produced by secondary hydrolysis. Planimetry of

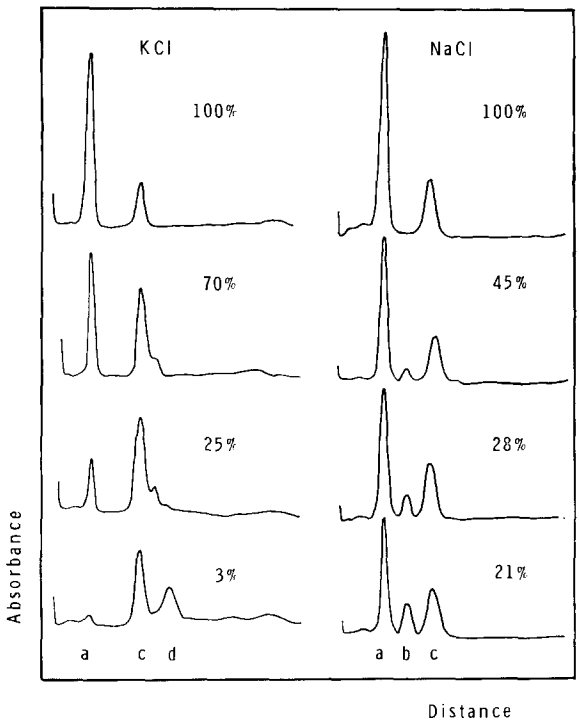

Fig. 5. Electrophoretograms of the proteins remaining in the membranes after tryptic digestion of purified (Na^+, K^+)-ATPase in presence of KCl (left) or NaCl (right). On each scan is given the activity of (Na^+, K^+)-ATPase remaining in percent of controls incubated without trypsin. To 25 μl of the resuspended sediments prepared as in Fig. 4 was added 25 μl 4 % sodium dodecyl sulfate, 2 % β-mercaptoethanol and samples were prepared for polyacrylamide gel electrophoresis in sodium dodecyl sulfate as described under Experimental. The gels were scanned at 600 nm in a Zeiss spectrophotometer with a Gilford linear transport attachment and an apperture of 0.1 mm.

the gel scans (Fig. 6) showed that the content of the large chain was reduced in proportion to the loss of (Na^+, K^+)-ATPase activity.

Digestion in presence of Na^+ caused remarkably few changes in the electrophoretic pattern in the initial phase of rapid inactivation of (Na^+, K^+)-ATPase (Fig. 5). The relative content of the large chain remained nearly constant (Fig. 6) and significant changes in the mobility of the large chain were not detectable. The precision of this determination was ± 2.5 % and it did therefore not exclude the possibility that smaller peptides were released from the large chain. In the final phase of inactivation, the large chain was cleaved to a fragment of molecular weight 78 000, fragment F_b (Fig. 5, peak b). Appearance of this fragment was accompanied by a decrease in the content of large chain (Fig. 6). Only small amounts of fragment F_c were formed with Na^+ in the medium, and fragments with molecular weights of 20–30 000 were not identified.

The gels stained with Coomasie blue did not show whether trypsin cleaved the smaller chain of the native enzyme because it had the same mobility in the gels as fragment F_c arising by cleavage of the large chain.

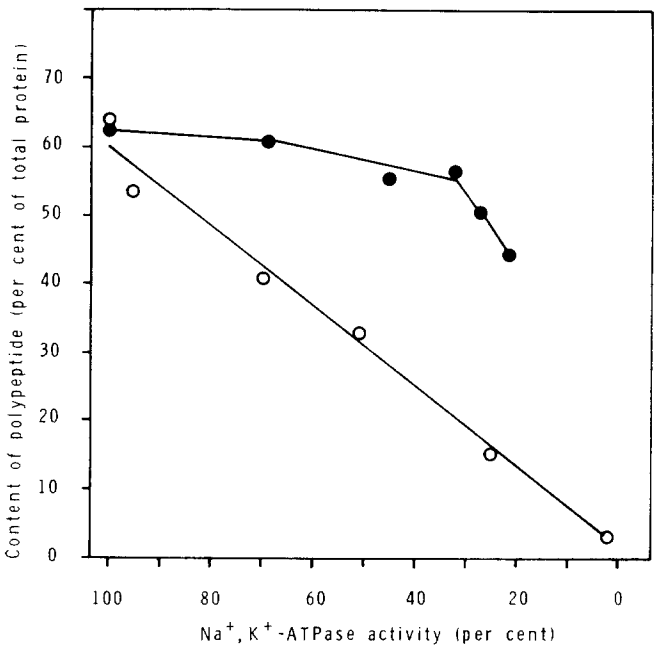

Fig. 6. Relationship between the content of large polypeptide in the membranes and the activity of (Na^+, K^+)-ATPase after digestion by trypsin in presence of KCl (○) and NaCl (●). Procedure as described in Fig. 5. The area under the peak of large chain in percent of the total area of the scan was determined by a Haff planimeter as described before [11].

The results in this section allowed distinction of two patterns of tryptic digestion of (Na^+, K^+)-ATPase seen with K^+ and Na^+ in the medium. Cleavage of the large chain in presence of K^+ was associated with linear inactivation curves suggesting that bonds were split which reacted with trypsin at identical rates. In agreement with the theoretical analysis of inactivation data [28], the biphasic kinetic pattern with Na^+ in the medium appeared to result from cleavage of two or more bonds which reacted with trypsin at different rates. The bonds cleaved in the initial faster phase were not located, but they were distinct from the bonds cleaved in the presence of K^+. In the second phase, the rate of cleavage of the large chain to fragment F_b was much lower than the rate of cleavage of the other bonds.

Effect of ATP and Mg · ATP on tryptic digestion of (Na^+, K^+)-ATPase in presence of K^+ and Na^+

The experiments in Figs 7 and 8 show that the patterns of tryptic digestion seen with K^+ and Na^+ in the medium were reversed by addition of ATP and Mg^{2+} plus ATP.

With 5 mM ATP and K^+ in the medium the inactivation curve was biphasic (Fig. 7), and Fig. 8 shows that the pattern of cleaved fragments was identical to that seen with Na^+ alone. Fragment F_b (mol. wt 78 000) appeared in the phase of slower inactivation and only traces of the smaller fragments were seen.

With Na^+ in the medium the inactivation curve became linear when 5 mM

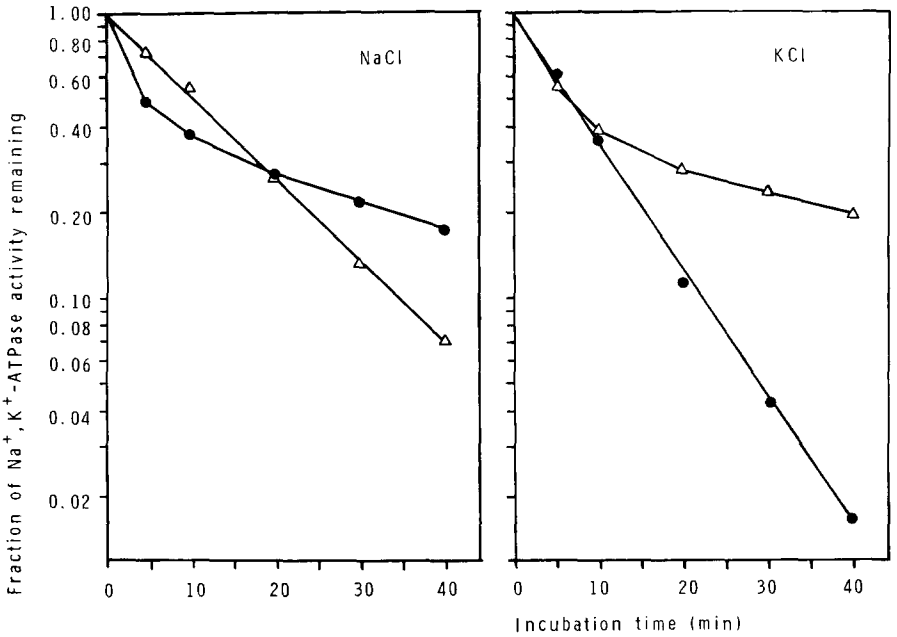

Fig. 7. Effect of Mg · ATP and ATP on tryptic inactivation of (Na^+, K^+)-ATPase in presence of NaCl and KCl. Incubation as in Fig. 1 in 1 ml containing 120 μg (Na^+, K^+)-ATPase protein, 9 μg trypsin, and 100 mM NaCl (●, left), 100 mM NaCl, 3 mM $MgCl_2$, 5 mM ATP (△, left), 100 mM KCl (●, right), and 100 mM KCl, 5 mM ATP (△, right). Mixing with trypsin inhibitor and assay of (Na^+, K^+)-ATPase as in Fig. 1.

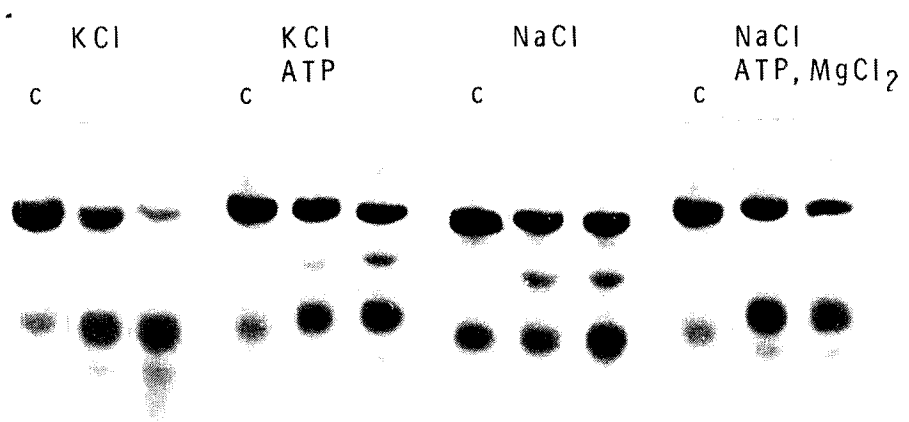

Fig. 8. Electrophoretic patterns of (Na^+, K^+)-ATPase digested with trypsin for 20 min and 40 min as described in Fig. 7. Procedure for sedimentation of the enzyme and for gel electrophoresis as described in Figs 4 and 5. c denotes gels of preparations incubated without trypsin.

ATP and 3 mM Mg^{2+} were added (Fig. 7). The large chain was cleaved to the fragments seen after digestion with K^+ alone in the medium and fragment F_b was no longer visible (Fig. 8).

The purpose of the experiments shown in Figs 9 and 10 was to analyze these effects of ATP on the kinetics of tryptic inactivation and to study the effect of ADP. It is seen from Fig. 9 that addition of ATP or ADP gave biphasic curves with K^+ in the medium. Mg^{2+} was not required and addition of Mg^{2+} did not alter the pattern (not shown). Addition of 0.1–1.0 mM ATP or 1–3 mM ADP decreased the rate of inactivation in the final phase of digestion whereas 2 and 4 mM ATP was required to increase the initial rate of inactivation. ATP was 3–4 times more potent than ADP as judged from the concentration dependence of the effect on the inactivation in the final phase and the concentration of ATP needed for half maximum effect was about 0.2 mM. This value is close to the apparent K_m for hydrolysis of ATP, and the relative potency of ATP and ADP is in good agreement with their relative affinity for the ATP binding site of (Na^+, K^+)-ATPase [29].

The experiments in Fig. 10 show that addition of both ATP and Mg^{2+} was required to alter the biphasic curve seen with Na^+ in the medium to a linear inactivation curve. Addition of ATP alone or of ADP plus Mg^{2+} did not change the inactivation curve, whereas addition of low concentrations of Mg^{2+} to a medium containing Na^+ and 1 mM ATP had a pronounced effect. The initial part of the curve became

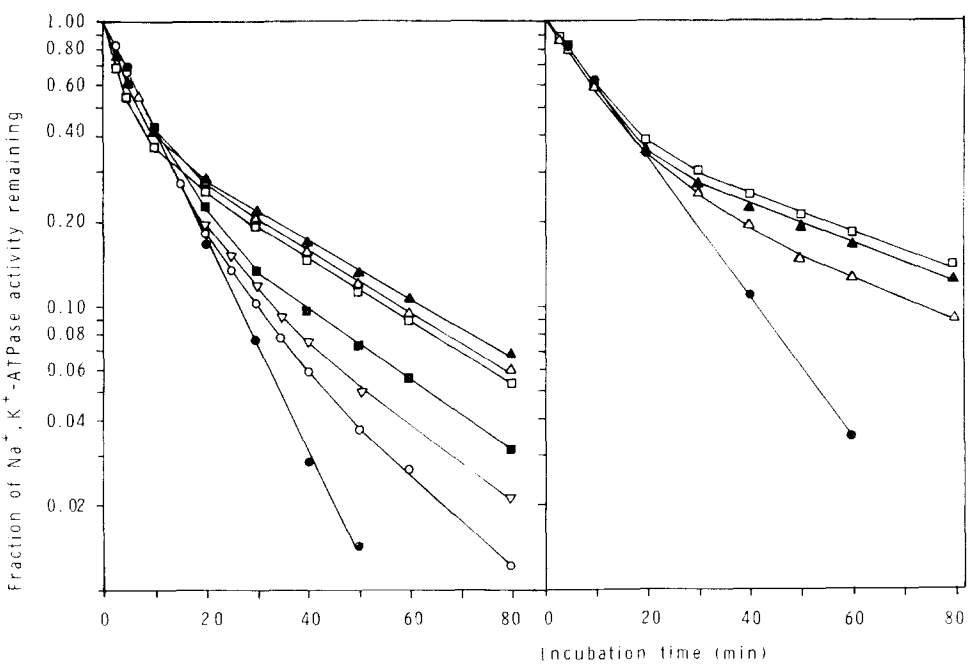

Fig. 9. Effect of ATP (left) and ADP (right) on tryptic inactivation of (Na^+, K^+)-ATPase in presence of KCl. Incubation as in Fig. 1 in 1 ml containing 100 μg (Na^+, K^+)-ATPase protein, 10 μg trypsin, 100 mM KCl and the following concentrations of ATP (left): 0 mM (●), 0.1 mM (○), 0.2 mM (▽), 0.3 mM (■), 1 mM (△), 2 mM (▲), and 4 mM (□), or the following concentrations of ADP (right): 0 mM (●), 1 mM (△), 2 mM (▲), and 3 mM (□). Mixing with trypsin inhibitor and assay of (Na^+, K^+)-ATPase as in Fig. 1.

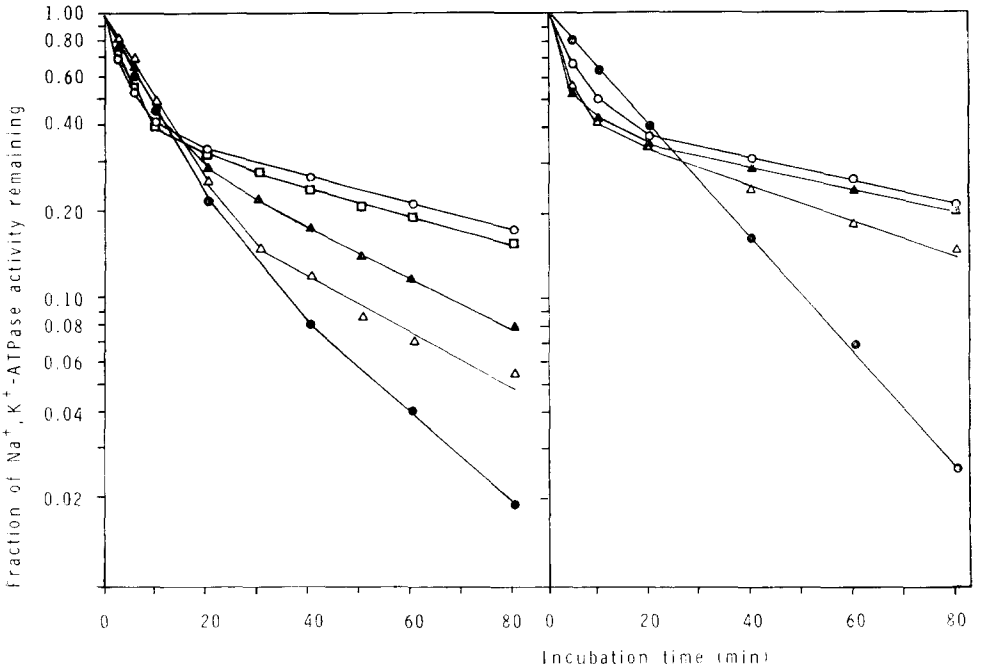

Fig. 10. Effect of ATP, ADP, and $MgCl_2$ on tryptic inactivation of (Na^+, K^+)-ATPase in presence of NaCl. Incubation as in Fig. 1 in 1 ml containing 100 μg (Na^+, K^+)-ATPase protein, 10 μg trypsin, 150 mM NaCl and (in the left part) 0 mM ATP, 0 mM $MgCl_2$ (○), 1 mM ATP, 0 mM $MgCl_2$ (□), 1 mM ATP, 0.05 mM $MgCl_2$ (▲), 1 mM ATP, 0.2 mM $MgCl_2$ (△), and 1 mM ATP, 1 mM $MgCl_2$ (●). In the experiments shown in the right part, the concentrations of ATP, ADP and $MgCl_2$ were: 0 mM ATP, 0 mM $MgCl_2$ (○), 2 mM ATP, 2 mM $MgCl_2$ (●), 0 mM ADP, 0 mM $MgCl_2$ (△), 3 mM ADP, 3 mM $MgCl_2$ (▲).

linear and the rate of inactivation in the final phase was increased. With 1 mM ATP and 1 mM Mg^{2+} the curve was linear until 80 % of the activity had been lost. The deflection of the curve in the final phase coincided with disappearance of ATP as measured by the release of P_i to the incubation medium. With 2 mM ATP and 2 mM Mg^{2+} the inactivation curve was linear and some ATP was left after complete inactivation. The hydrolysis of ATP prevented measurements of the concentration dependence for the effect of ATP in presence of Mg^{2+} and Na^+.

Somogyi [5] found no difference in the effect of Na^+ and K^+ on the tryptic inactivation of (Na^+, K^+)-ATPase and concluded that the protection by K^+ is lessened by ATP and that ATP is without effects in presence of Na^+ and Mg^{2+}. The disagreement with our results is readily explained by differences in technique. Somogyi [5] measured the activity of (Na^+, K^+)-ATPase after incubation of a rat brain preparation with trypsin for 20 min, most often with 50–80 % of the activity remaining. The present results show that analysis of the effects of ions and ATP requires that the time course of inactivation is followed.

Tryptic digestion of the purified large chain

The large chain of (Na^+, K^+)-ATPase can be purified by gel chromatography

in sodium dodecyl sulfate. After complete removal of sodium dodecyl sulfate by a resin [30] the protein is water soluble. The preparation is devoid of lipid and has no catalytic activity [31, 32]. Fig. 11 shows that this protein was degraded rapidly by trypsin to small peptides and that the pattern was the same whether Na^+ or K^+ was present. Some of the peptides may have left the gels by diffusion. Most of the remaining peptides migrated with the marker, but a weak band with a molecular weight of about 15 000 was seen.

This result could be expected because lysin and arginine form about 10 % of the amino acid residues of the large chain [31]. The average tryptic peptide should thus contain about 10 residues. It is also known that denatured proteins are more susceptible to proteolysis than native proteins [21].

Fig. 11. Electrophoretic patterns of purified large chain (mol. wt 98 000) incubated without trypsin (A), and of large chain digested with trypsin for 10 min in presence of KCl (B) and NaCl (C). The water-soluble, large chain was prepared as before [32]. Aliquots containing 50 μg protein were incubated in 0.1 ml at 37 °C with 25 mM imidazole, pH 7.5, 1.25 μg trypsin and 100 mM KCl or 100 mM NaCl. After 10 min, 40 μl of this medium was mixed with 10 μl 8 % sodium dodecyl sulfate, 4 % β-mercaptoethanol. The mixture was heated at 100 °C for 3 min and samples were prepared for gel electrophoresis. The proteins used for gel A were incubated with 100 mM KCl, but without trypsin.

Control experiments

In view of the results described above it was important to determine the activity and the stability of trypsin in the media used in the experiments. Table I shows that both the esterase activity and the proteolytic activity of trypsin was slightly higher with Na^+ than with K^+ in the medium. Both activities were lower than with

TABLE I

ESTEROLYTIC AND PROTEOLYTIC ACTIVITY OF TRYPSIN IN MEDIA CONTAINING NaCl, KCl, $MgCl_2$ AND ATP

The esterolytic assay [21] was performed at 37 °C in 2 ml 30 mM Tris, pH 7.5, with 1 mM *p*-toluenesulfonyl-L-arginine methyl ester and either 150 mM NaCl, or 150 mM KCl, or 120 mM choline chloride, 10 mM $CaCl_2$. The absorbance was read at 247 nm and the reaction was started by addition of trypsin to final concentrations of 0.16, 0.32, and 0.63 $\mu g \cdot ml^{-1}$. The rate of increase of absorbance was linear and proportional to the concentration of trypsin.

Determination of proteolytic activity [21] was done at 37 °C in 1 ml 25 mM imidazole, pH 7.5, with 0.5 mg casein per ml and 100 mM NaCl or 100 mM KCl or 10 mM $CaCl_2$, 70 mM choline chloride or 100 mM KCl, 5 mM ATP or 100 mM KCl, 5 mM ATP, 3 mM $MgCl_2$. The reaction was started by addition of 1.0 μg trypsin. The amount of released peptides was measured as described in Experimental after 5, 10, and 20 min of incubation and the activity was calculated from linear plots of the rate of digestion using standard activity curves for trypsin according to Kunitz [20].

	Esterolytic activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Proteolytic activity $((TU)^{cas})$*
NaCl	217	0.0040
KCl	193	0.0037
$CaCl_2$, choline chloride	240	0.0048
KCl, ATP		0.0039
NaCl, ATP, $MgCl_2$		0.0042

* $(TU)^{cas}$ is the activity which gives rise to an increase of one unit of absorbance at 280 nm per min digestion [20].

TABLE II

RATES OF INACTIVATION OF THE ESTEROLYTIC AND PROTEOLYTIC ACTIVITIES OF TRYPSIN IN MEDIA CONTAINING NaCl, KCl, $MgCl_2$, AND ATP

Inactivation of the esterolytic activity was measured by incubating 2.5 μg trypsin at 37 °C in 1 ml 25 mM imidazole, pH 7.5, with 150 mM NaCl, or 150 mM KCl, or 10 mM $CaCl_2$, 120 mM choline chloride. Inactivation of the proteolytic activity was measured by incubating 10 μg trypsin in 1 ml of the media used for the proteolytic assays in Table I without casein. After incubation for 0, 10, 20, 30, 40, 60, and 80 min, 0.1 ml of these media was transferred for assay of esterolytic or proteolytic activity as described in Table I with $CaCl_2$ and choline chloride in the assay medium. The values for rate constants were calculated from linear first-order plots of the fraction of original activity remaining at the various incubation times.

	Loss of esterolytic activity (min^{-1})	Loss of proteolytic activity (min^{-1})
NaCl	0.011	0.012
KCl	0.012	0.013
$CaCl_2$, choline chloride	0.0015	0.0017
KCl, ATP		0.016
NaCl, ATP, $MgCl_2$		0.015

Ca^{2+}, which is known to activate trypsin [21]. Only the proteolytic activity could be assayed with ATP in the medium due to its absorption of ultraviolet light. It is seen that addition of ATP and of ATP plus Mg^{2+} had almost no effect on the activity of trypsin. Table II shows that the rate of inactivation of trypsin was the same with Na^+ or K^+ or with combinations of the ions and ATP in the medium. Expectedly, Ca^{2+} protected against inactivation of trypsin, probably by retarding autolysis [21], but Ca^{2+} was not added in the experiments because it is an inhibitor of (Na^+, K^+)-ATPase. It can be concluded from these experiments that the ions and ATP are without specific effects on the activity or the stability of trypsin that can explain their effects on the digestion of (Na^+, K^+)-ATPase.

DISCUSSION

Tryptic digestion of the large chain of the purified, membrane-bound (Na^+, K^+)-ATPase is slow and rather specific, whereas the water-soluble, lipid-free preparation of the large chain is split rapidly to numerous small fragments. A significant proportion of the protein seems therefore to be embedded in the membrane and protected from trypsin binding and only a few of the L-lysyl or L-arginyl bonds are exposed on the membrane surface in positions favourable to trypsin attack.

The differences in both the inactivation kinetics and the cleavage of the large chain can in theory be due to effects of the ions and ATP on the specificity of trypsin. The control experiments and the cation specificity in part exclude this possibility. It is also unlikely that ATP should alter the specificity of trypsin in a way which depends on Na^+, K^+, and Mg^{2+}. It can therefore be concluded that the regions of the large chain exposed to reaction with trypsin in the presence of K^+ or Na^+, Mg^{2+} and ATP are distinct from the regions exposed in presence of Na^+ or K^+ and ATP. This means that the structure of the large chain or its position in the membrane is altered and that two conformations of (Na^+, K^+)-ATPase, termed the (t)K-form and the (t)Na-form, can be defined by their characteristic patterns of tryptic digestion.

The results do not show whether the conformational changes result from an altered protein structure or from interaction of different regions of the protein with lipids or carbohydrate components of the membrane. The previous use of trypsin as a probe for protein structure does not help in distinguishing between these possibilities. It was shown that trypsin can sense distinct structural regions of protein molecules [12] and conformational changes in enzyme proteins [13] as well as specific interactions of a membrane protein with lipids [16].

The conformational changes can be discussed in terms of the following equilibria:

$$(t)E_P(K) \overset{Na^+}{\underset{K^+}{\rightleftharpoons}} (t)E_S(Na) \qquad (1)$$

$$(t)E_P(K) \overset{ATP}{\rightleftharpoons} (t)E_S(K \cdot ATP) \qquad (2)$$

$$(t)E_S(Na \cdot ATP) \overset{Mg^{2+}}{\rightleftharpoons} (t)E_P(Na^P_{ADP}) \qquad (3)$$

The transitions between the (t)K-form and the (t)Na-form (Reaction 1) on exchanging K^+ and Na^+ show that (Na^+, K^+)-ATPase undergoes conformational changes

resulting from binding of Na^+ and K^+ in the absence of other ligands. This conclusion is in agreement with models based on kinetic data in which it is proposed that the enzyme exists in equilibrium between a Na-form and a K-form [17] and that binding of Na^+ and K^+ alter the equilibrium between the conformations [34]. The enzyme has a high affinity for K^+ relative to Na^+ in the absence of ATP [17, 35]. ATP binds easily to the Na-form and with difficulty to the K-form [33, 36] and ATP shifts the equilibrium toward the Na-form [23, 24].

Transition from the (t)K-form to the (t)Na-form in presence of K^+ (Reaction 2) requires relatively high concentrations of ATP. ADP has a similar effect and Mg^{2+} is not necessary. These requirements are similar to those found in kinetic studies for an activating effect of ATP on an occluded conformation of the site for translocation of K^+ [37]. This effect of ATP is distinct from its role as phosphate donor and it gives an increase in the affinity for Na^+ relative to K^+ [38]. The present results show that this change in affinity is accompanied by reversal to the conformation occurring with Na^+ alone in the medium.

The requirements for transition from the (t)Na-form to the (t)K-form in presence of Na^+ (Reaction 3) are identical to those for phosphorylation of the large chain from ATP [17, 36]. Titration with *N*-ethylmaleimide shows that sulfhydryl groups within the large chain are exposed on phosphorylation [39]. The present results support the conclusion that this reflects a conformational change in the protein [40].

The reactivity of the sulfhydryl groups to *N*-ethylmaleimide is not altered by addition of ATP in the presence of K^+ [23, 39] and there is only a small change in reactivity when K^+ is exchanged for Na^+ in the absence of ATP [23]. The conformational changes sensed with trypsin under these conditions (Reactions 1 and 2) are therefore not detectable with *N*-ethylmaleimide.

The sulfhydryl groups within the large chain are protected when K^+ is exchanged for Na^+ in the presence of ATP [23, 39], whereas the patterns of tryptic digestion in presence of ATP plus K^+ and ATP plus Na^+ are similar. The protection of sulfhydryl groups may thus represent a change in protein structure which is detectable with *N*-ethylmaleimide but not with trypsin. The explanation for this can be that it is a subtle change or that the size and specificity of the two probes differ considerably. This illustrates that complete mapping of the conformational changes in (Na^+, K^+)-ATPase requires examination with different probes of the structure of membrane proteins.

Incorporation of the changes in conformation detected with trypsin in a model for the hydrolysis of ATP by (Na^+, K^+)-ATPase requires modification of previous schemes [17, 36, 41].

$$\underset{}{K\text{-}E_2} + ATP \rightleftharpoons \underset{(a)}{K\text{-}E_1\text{-}ATP} \overset{Na^+}{\underset{K^+}{\rightleftharpoons}} \underset{(b)}{Na\text{-}E_1\text{-}ATP}$$

$$\underset{(c)}{Na\text{-}E_1\text{-}ATP} \overset{Mg^{2+}}{\rightleftharpoons} \underset{(d)}{Na\text{-}E_{2\text{-}ADP}^{\sim P}} \overset{K^+}{\underset{Na^+}{\rightleftharpoons}} K\text{-}E_2 + ADP + P_i$$

In this hypothetical scheme E_1 corresponds to the (t)Na-form and E_2 to the (t)K-form. It is assumed that reaction of a potassium-enzyme complex with ATP forms the

initial step. In addition to Reaction 2 (step a) and Reaction 3 (step c) the scheme contains steps in which the (t)Na-form exchanges K^+ for Na^+ (step b) and the (t)K-form exchanges Na^+ for K^+ (step d). The kinetic data mentioned above suggest that the conformational changes are accompanied by shifts in the affinities for Na^+ and K^+. It is therefore possible that the ion exchange reactions can occur at concentrations of Na^+ and K^+ which are optimal for ATP hydrolysis. The rates of step a will depend on the ATP concentration and the rates of steps b and d on the Na^+/K^+ ratio and any of these steps can be rate limiting. It can be assumed that Na-E_1-ATP is transient in the presence of Mg^{2+}. The steady-state level of phosphoenzyme will then depend on the relative rates of the reactions in steps b and d. Masking and exposure of sulfhydryl groups occur in steps b and c.

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