Biochimica et Biophysica Acta, 466 (1977) 97—108

BBA 77657

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

VI. DIFFERENTIAL TRYPTIC MODIFICATION OF CATALYTIC FUNCTIONS OF THE PURIFIED ENZYME IN PRESENCE OF NaCl AND KCl

PETER LETH JØRGENSEN

With the technical assistance of JANNE PETERSEN

Institute of Physiology, University of Aarhus, Aarhus (Denmark)

(Received September 29th, 1976)

Summary

1. Two distinct patterns of tryptic modification of the catalytic functions of purified (Na^+ + K^+)-ATPase can be related to the two previously described patterns of enzyme inactivation and cleavage of the large chain seen with NaCl and KCl (Jørgensen, P.L. (1975) Biochim. Biophys. Acta 401, 399—415).

2. With NaCl, in phase A, the rapid inactivation of 50—55% of the (Na^+ + K^+)-ATPase activity is associated with loss of 85% of the K^+-phosphatase activity and an increase in Na^+-ADP-ATP exchange activity to 150% of control. ATP binding and phosphorylation are unchanged and the inactivation may result from cleavage of bonds within the large chain which are involved in dephosphorylation reactions. In phase B with NaCl, ATP binding and phosphorylation are lost slowly in parallel to inactivation of (Na^+ + K^+)-ATPase and cleavage of the large chain to a fragment with M_r = 78 000.

3. With KCl, cleavage of the large chain to almost equal fragments abolish ATP binding and phosphorylation in parallel to the inactivation of (Na^+ + K^+)-ATPase. An additional split seems required for inactivation of the K^+-pNPPase activity.

4. After completion of the digestion in phase A with NaCl a stable preparation can be isolated in which the activity of (Na^+ + K^+)-ATPase is 40%. ATP binding and phosphorylation are 90%, K^+-phosphatase is 15%, and Na^+-ADP-ATP exchange is 150% of control. We currently examine if these levels are related to changes in phosphorylation kinetics.

5. The ATP binding area is much more stable to trypsin with NaCl than with KCl, but loss of the binding capacity is in both cases correlated to a distinct cleavage of the large chain. The relationship between the fractional loss of ATP binding and cleavage of the large chain suggests that the nucleotide binding area

is confined to one of the two large chains in the protein complex with M_r = 270 000 which binds one molecule of ATP.

6. The data also suggest that the phosphatase site is remote from the ATP binding area. It is proposed that the protein complex with M_r = 270 000 contains two large chains with different catalytic functions and that each chain forms a cation channel.

Introduction

Graded trypsinolysis of the purified membrane-bound ($Na^+ + K^+$)-ATPase provides a tool for detection of two conformational states in the catalytic protein of the enzyme, a Na^+ form and a K^+ form [1]. Tryptic cleavage of the catalytic protein is specific [1,2] and the bonds exposed to digestion in the presence of KCl are distinct from those exposed in the presence of NaCl reflecting that binding of Na^+ and K^+ in absence of other ligands cause transitions between a Na^+ form and a K^+ form of ($Na^+ + K^+$)-ATPase [1].

Binding sites and catalytic areas are likely to be exposed on the membrane surface and have been shown to be modified by tryptic digestion [1—3]. Differences in susceptibility of catalytic functions to trypsin depending on the presence of NaCl and KCl could provide information about the localization of catalytic sites within the catalytic protein of ($Na^+ + K^+$)-ATPase and about the nature of the transition between the Na^+ form and the K^+ form.

The purpose of the present work has therefore been to determine the time course of the tryptic modification of the capacities for ATP binding and sodium-dependent phosphorylation, the phosphatase activity and the ADP-ATP exchange activity of the purified ($Na^+ + K^+$)-ATPase with KCl or NaCl in the medium. Modification of these catalytic functions are related to the two different patterns of tryptic cleavage of the large chain and inactivation of ($Na^+ + K^+$)-ATPase which are observed in the presence of KCl and NaCl [1].

Experimental

($Na^+ + K^+$)-ATPase was purified 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 [4,5]. Treatment with trypsin (Sigma, twice crystallized, salt free) was carried out at 37° C in 25 mM imidazole buffer, pH 7.5, with 150 mM NaCl or 150 mM KCl and 100 μg ($Na^+ + K^+$)-ATPase protein per ml. A controlled and reproducible rate of digestion was obtained by addition of 1 I.U. trypsin per 100 μg enzyme protein. Trypsin activity was determined by the esterase assay [6] and 1 I.U. hydrolyzed 1 μmol *p*-toluenesulfonyl-L-arginine methyl ester per min at 37° C and corresponded to about 4 μg protein. Digestion was stopped by mixing with Soyabean trypsin inhibitor (Sigma type I-S) to a weight ratio of inhibitor to trypsin of 3—4 : 1. The membranes were spun down by centrifugation for 120 min at 50 000 rev./min in the Beckmann type 65 rotor and were washed once in 25 mM imidazole, pH 7.5, 150 mM NaCl and twice in 25 mM imidazole, pH 7.5. Aliquots were taken out after resuspension for ($Na^+ + K^+$)-ATPase assay,

protein analysis and polyacrylamide gel electrophoresis in sodium dodecyl sulfate as before [1]. Determination of ATP binding, phosphorylation, ADP-ATP exchange and phosphatase activity was done as before [7,8]. Details are given in the figure legends.

Results

Cleavage of the large chain

The time courses of tryptic inactivation of $(Na^+ + K^+)$-ATPase and the electrophoretic patterns of native and cleaved proteins after digestion in presence of NaCl or KCl were described before [1]. Inactivation curves are also shown in Figs. 3, 5 and 7. Figs. 1 and 2 provide a more detailed account of the relationship between the activity of $(Na^+ + K^+)$-ATPase and the content of large chain and cleaved fragments after digestion in presence of NaCl or KCl.

Fig. 1 shows that the loss of $(Na^+ + K^+)$-ATPase activity in presence of KCl was proportional to cleavage of the large chain to fragments of M_r = 58 000 and 46 000. Later a smaller fragment with M_r = 38 000 was formed by secondary hydrolysis.

With NaCl, there were two phases of digestion, phase A in which 50—55% of the $(Na^+ + K^+)$-ATPase activity was lost at a high inactivation rate and phase B in which 45—50% of the activity was lost at a 15—20-fold lower rate. (cf. ref. 1 and Figs. 3, 5 and 7). The cleavage responsible for the inactivation in phase A

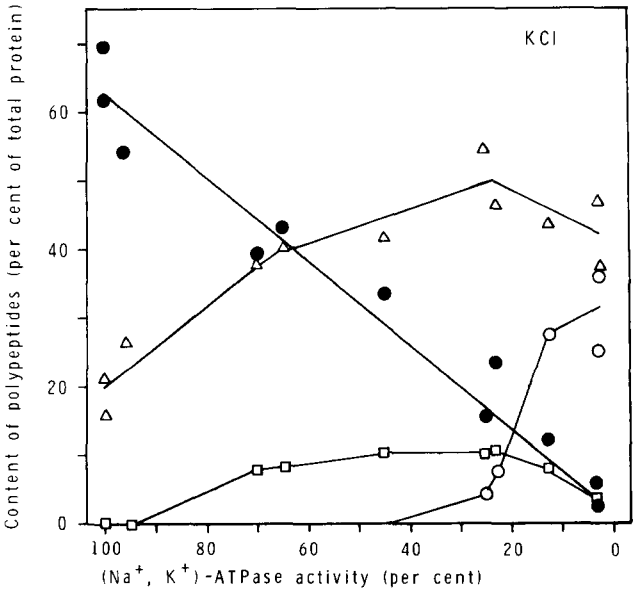

Fig. 1. Relationship between the content of polypeptides in the membranes and the activity of $(Na^+ + K^+)$-ATPase after tryptic digestion in presence of 150 mM KCl. Digestion was stopped after varying intervals and the preparations were centrifuged as described under Experimental. Assay of $(Na^+ + K^+)$-ATPase was done as before [1]. The polyacrylamide gels were stained with Coomassie blue and scanned at 600 nm as before [1]. The area under the peak of each protein in percent of the total area of the scan was determined by a Haff planimeter. The molecular weights of the proteins as determined by calibration against known standards as before [1] were: •, 99 000; △, 58 000; □, 46 000; and ○, 38 000.

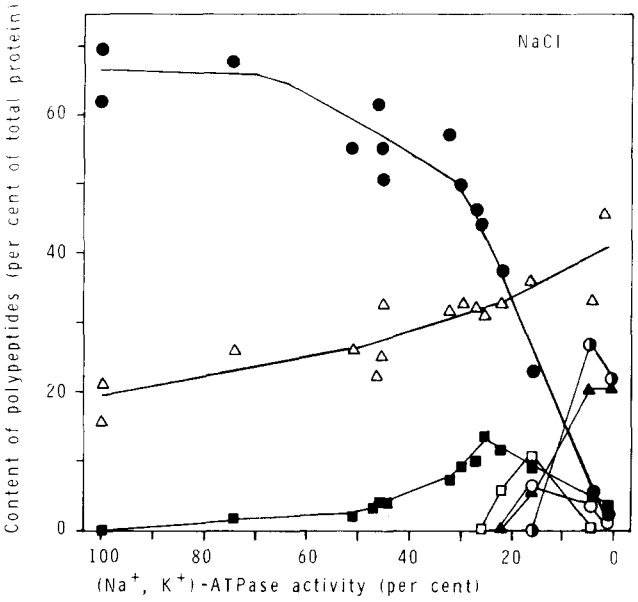

Fig. 2. Relationship between the content of polypeptides in the membranes and the activity of $(Na^+ + K^+)$-ATPase after tryptic digestion in presence of 150 mM NaCl. Procedure as in Fig. 1. Molecular weights of the proteins were: ●, 98 000; ■, 78 000; △, 58 000; □, 44 000; ○, 37 000; ▲, 23 000; ◑, 15 000.

has not been identified and Fig. 2 shows that the relative content of the catalytic protein remained nearly constant. In phase B there was a linear relationship between the loss of $(Na^+ + K^+)$-ATPase activity and cleavage of the large chain to the fragment with $M_r = 78\,000$. Minor fragments of $M_r = 44\,000$, 37 000, 23 000, and 15 000 were formed by secondary hydrolysis.

The data in Figs. 1 and 2 show that there was a reproducible relationship between inactivation of $(Na^+ + K^+)$-ATPase and cleavage of the large chain in the presence of NaCl or KCl. In the following experiments, the determinations of $(Na^+ + K^+)$-ATPase activity therefore served to establish a relationship between modification of the catalytic functions and cleavage of the protein.

Tryptic modification of catalytic functions

(a) *ATP binding.* The semilogaritmic plots in Fig. 3 show that tryptic digestion in the presence of KCl reduced the capacity for binding of ATP at a 6—8-fold faster rate than did digestion in the presence of NaCl. With KCl, ATP binding was reduced in parallel to cleavage of the large chain to fragments with $M_r = 58\,000$ and 46 000 and to the inactivation of $(Na^+ + K^+)$-ATPase.

With NaCl, ATP binding was not affected by the cleavage causing the rapid loss of $(Na^+ + K^+)$-ATPase activity in phase A, Fig. 3. The rate constant of the reduction in ATP binding capacity was low, $k = -0.0031\ min^{-1}$, and close to the rate constant of inactivation of $(Na^+ + K^+)$-ATPase in phase B.

The Scatchard plots in Fig. 4 show that the digestion reduced the capacity for binding of ATP without affecting the apparent affinity for ATP. The data of ATP binding to control preparations and to preparations which had been digested with trypsin in the presence of KCl and NaCl fell on straight lines with

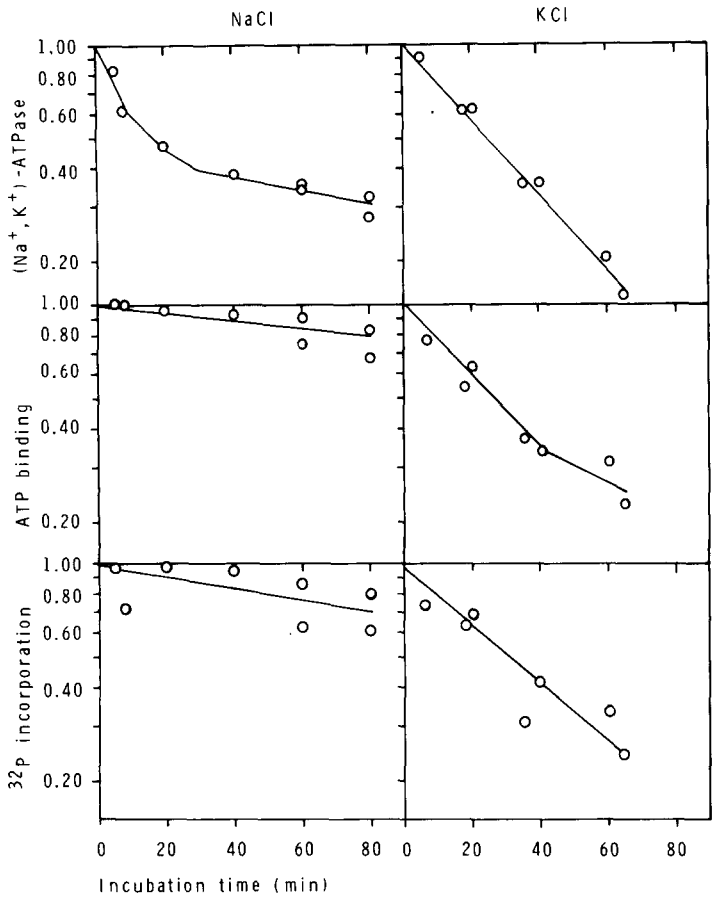

Fig. 3. Semilogaritmic plots of tryptic modification of ATP binding and sodium-dependent phosphorylation and inactivation of $(Na^+ + K^+)$-ATPase in presence of 150 mM NaCl (left) or 150 mM KCl (right). Aliquots each containing 600 μg protein were incubated with trypsin. Digestion was stopped at the indicated intervals and the membranes were spun down and washed as described under Experimental. Aliquots were taken out for assay of $(Na^+ + K^+)$-ATPase as in Fig. 1, and for determination of ATP binding as in Fig. 4 using a total ATP concentration of 1.3 μM. For determination of sodium-dependent phosphorylation portions containing 100 μg enzyme protein were incubated for 4 s at 37°C with 30 μM [γ-^{32}P]ATP in 3 ml 3 mM $MgCl_2$, 30 mM Tris · HCl, pH 7.4 (37°C) and 100 mM NaCl or 100 mM KCl. The reaction was stopped with $HClO_4$, and the protein was separated by centrifugation and washed as before [7]. The phosphorylation level was taken to be the difference between the incorporation of ^{32}P with NaCl and the incorporation with KCl.

slopes that did not differ significantly. The dissociation constants were 0.079—0.11 μM. The control curves show that K^+ was effectively removed by the repeated washings after the incubation, since it is known that even low concentrations of K^+ increase the dissociation constant of the enzyme-ATP complex [9].

The data in Fig. 4 yielded a maximum capacity for binding of ATP to the control preparations of 3.7 ± 0.1 nmol per mg protein corresponding to a molecular weight of 270 000 per ATP binding site. This value is in agreement with the previous estimate for the enzyme unit binding one molecule of ATP or ouabain [7].

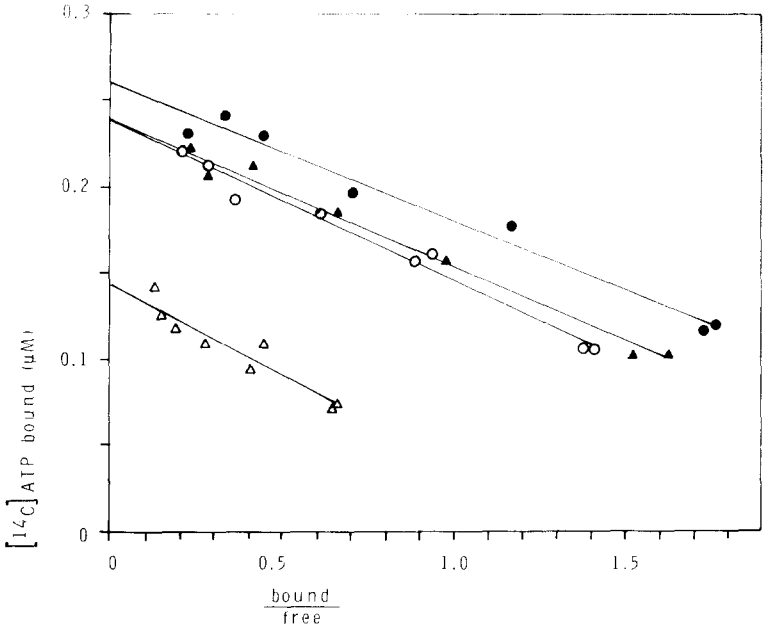

Fig. 4. Scatchard plot of ATP binding to four preparations of $(Na^+ + K^+)$-ATPase. Two control preparations were incubated without trypsin in presence of (●) NaCl and (▲) KCl. The two other preparations were incubated with trypsin and (○) NaCl or (△) KCl until the $(Na^+ + K^+)$-ATPase activity had decreased to 39 and 42% of control, respectively. The membranes were spun down and washed as described under Experimental. Aliquots of the resuspended sediments were mixed in centrifuge tubes at 0°C in a total volume of 1 ml 30 mM Tris/EDTA, pH 7.5, with [^{14}C]ATP and unlabelled ATP to total concentrations varying from 0.2 to 1.3 μM. The bound and free ATP was determined after centrifugation as before [7].

(b) *Phosphorylation.* Tryptic digestion in the presence of NaCl or KCl reduced the maximum level of sodium-dependent phosphorylation at rates which were identical to the rate of reduction of ATP binding, Fig. 3. The reduction in phosphorylation capacity may therefore be secondary to the loss of the ability to bind ATP.

The maximum level of sodium-dependent phosphorylation was 3.6 ± 0.2 nmol per mg protein and similar to the ATP binding capacity. This value is 2-fold lower than the maximum phosphorylation level reported in a previous paper in this series [7]. The previously reported value was wrong and due to an error in calculation of the specific activity of [γ-^{32}P]ATP. After this correction the level of phosphorylation corresponds to one phosphorylation site per site for binding of ATP or ouabain.

Erdmann and Schoner [3] found that tryptic digestion in presence of 25 mM Tris reduced the $(Na^+ + K^+)$-ATPase activity to 20—30% whereas the binding sites for ADP had decreased only to 72% of control and phosphorylation was unchanged. This dissociation resembles our results after digestion in the presence of NaCl. We have not examined modification of catalytic functions in the presence of Tris, but the resemblance is expected because the biphasic pattern of inactivation of $(Na^+ + K^+)$-ATPase with NaCl is seen also with Tris and choline in the medium [1].

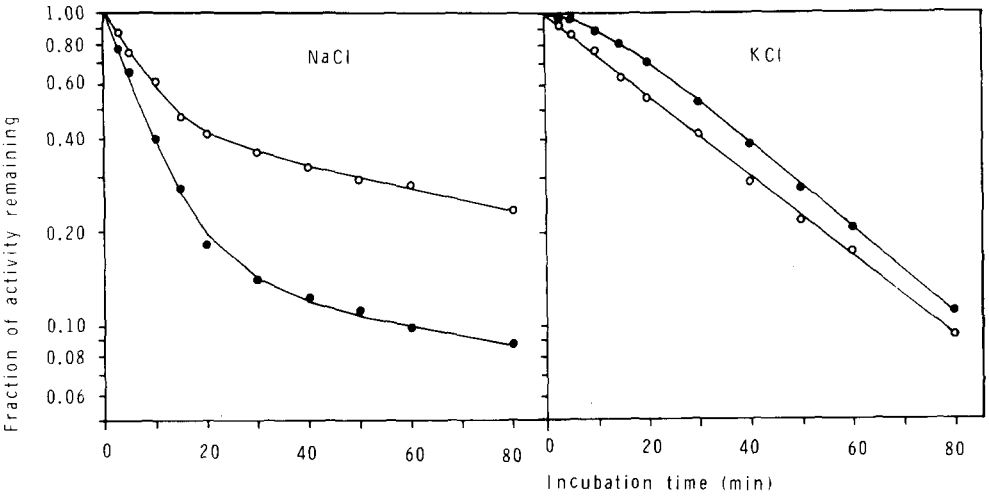

Fig. 5. Semilogaritmic plots of tryptic inactivation of (●) K^+-*p*-nitrophenylphosphatase activity and (○) ($Na^+ + K^+$)-ATPase activity in presence of 150 mM NaCl (left) or 150 mM KCl (right). Digestion was started by mixing 1.5 I.U. trypsin and 150 μg enzyme protein in a total volume of 1500 μl 25 mM imidazole, pH 7.5, 150 mM NaCl. At the indicated intervals, 100 μl of the incubation medium was mixed with 40 μl imidazole, 25 mM, pH 7.5, containing 1 μg trypsin inhibitor. After completion of the incubation with trypsin, 25 μl of this mixture was transferred to test tubes for assay of ($Na^+ + K^+$)-ATPase and K^+-phosphatase activity as before [1,4].

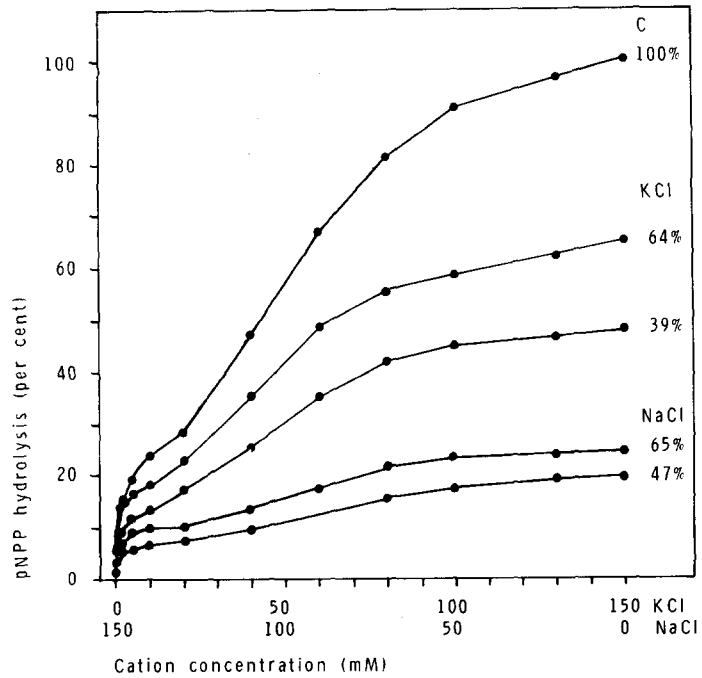

Fig. 6. Effect of $Na^+ + K^+$ on the *p*-nitrophenylphosphatase (pNPP) activity remaining after tryptic digestion in presence of 150 mM KCl or 150 mM NaCl. The ($Na^+ + K^+$)-ATPase activity of the control incubated without trypsin (C, 100%) and the activity remaining after digestion with KCl (K^+, 64% and 39%) or NaCl (65% and 47%) are shown to the right. Assay of ($Na^+ + K^+$)-ATPase as in Fig. 1 and of *p*-nitrophenylphosphatase as in Fig. 5.

(c) *The phosphatase activity.* Figs. 5 and 6 show that the patterns of tryptic inactivation of the K^+-phosphatase activity in the presence of either NaCl or KCl was significant different from those described for the ($Na^+ + K^+$)-ATPase activity [1].

With NaCl, the time courses of inactivation of both the K^+-phosphatase and the ($Na^+ + K^+$)-ATPase activities were biphasic. Fig. 5 shows that the measured points fit with the curves for the equation $EA/EA_0 = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$ in which EA is the activity remaining at time t; A and B are the fractions of the activity in phase A and B, and α and β are the respective rate constants. It is seen that a very large fraction of the K^+-phosphatase activity, 0.85, was lost in phase A as compared with 0.53 for the ($Na^+ + K^+$)-ATPase activity. The rate constant, α, was -0.122 min^{-1} for both enzyme activities. The fractions of the activities lost in phase B were 0.15 for the K^+-phosphatase and 0.47 for the ($Na^+ + K^+$)-ATPase and the rate constants (β) were 18- and 15-fold smaller, respectively, than the rate constants (α) of phase A.

Extrapolation to longer times shows that the phosphatase and the ATPase activities approached zero simultaneously, and the data suggest that neither of the two activities remains after inactivation of the other.

With KCl, the initial rate of inactivation of K^+-phosphatase was very low, Fig. 5. Later the rate constant was increased in a negative sense and approached the value for the rate constant for the linear time course of inactivation of ($Na^+ + K^+$)-ATPase ($k = -0.0298$ min^{-1}). Fig. 6 shows that the data of inactivation of the K^+-phosphatase activity fit the curve for the equation $EA/EA_0 = \exp(-k_2 t) + \exp(-k_1 t) - \exp[-(k_1 + k_2)t]$, ($k_1 = -0.0297$, $k_2 = -0.515$ min^{-1}) which describes the time course of enzyme inactivation when modification of two residues is required for elimination of activity (ref. 10, case II).

This observation suggests that cleavage of two bonds is required for tryptic inactivation of the K^+-phosphatase activity in presence of KCl. However, only one split cleaving the large chain to fragments of 58 000 and 46 000 has been identified. Similarly, the bond cleaved in phase A of tryptic digestion in the presence of NaCl cannot be identified by sodium dodecyl sulfate gel electrophoresis. It seems possible that the inactivation of the K^+-phosphatase activity both in phase A with NaCl and in the presence of KCl is due to cleavage of a bond close to the terminal end of the catalytic protein. The split may release a fragment which is too small for detection by sodium dodecyl sulfate gel electrophoresis. This bond appears to be exposed and rapidly cleaved in presence of NaCl. With KCl, it appears to be protected and to become exposed after cleavage of the catalytic protein in two almost equal fragments.

The tryptic inactivation of the K^+-phosphatase activity with KCl or NaCl in the medium was not due to a change in affinity constants. The shape of the curves relating phosphatase activity to the Na^+/K^+ ratio was not altered by tryptic digestion, Fig. 6. In all cases the activity was maximal with 150 mM KCl and the ratio of the activity in the digested preparations to the activity of the control preparations was the same in the range of 40—150 mM KCl. At lower K^+/Na^+ ratios the depression of the activity was slightly smaller for preparations digested in the presence of NaCl suggesting that Na^+ is less inhibitory for these preparations.

The ratio of the activity of control preparations to the activity in prepara-

tions digested for 40 min in the presence of NaCl and KCl was independent of the concentration of *p*-nitrophenylphosphate in the range from 0.1 to 20 mM at a constant Mg^{2+} concentration of 20 mM. The substrate concentrations for half maximum activity were within the range of 2.5—3.3 mM (not shown, cf. ref. 11).

At a low K^+/Na^+ ratio ATP stimulates the phosphatase activity and a new level is reached at 0.05—0.1 mM ATP [11]. ATP caused a 2-fold increase in activity for all preparations. The stimulation by ATP and the ratio between the K^+-phosphatase and the ($Na^+ + K^+$ + ATP)-phosphatase activity were thus not affected by tryptic digestion in the presence of NaCl or KCl (not shown).

(d) *ADP-ATP exchange activity.* The purified preparation catalyzed a Na^+-dependent and a ($Na^+ + K^+$)-dependent (^{14}C)-ADP-ATP exchange reaction [8]. The time course of the tryptic modification of the (Na^+)-ADP-ATP exchange was entirely different from modification of the ($Na^+ + K^+$)-ADP-ATP exchange activity. In the presence of NaCl (Fig. 7), tryptic digestion in phase A led to a 50—60% increase in the (Na^+)-ADP-ATP exchange activity. In phase B, with Na^+, the exchange activity was reduced at the same rate as the ($Na^+ + K^+$)-ATPase activity. With KCl (Fig. 7), the initial rate of inactivation of the (Na^+)-ADP-ATP exchange activity was very low, but later the rate was increased. This kinetic pattern resembles that observed for the phosphatase activity, but the data are too scarce for exact evaluation. Tryptic digestion with NaCl or KCl

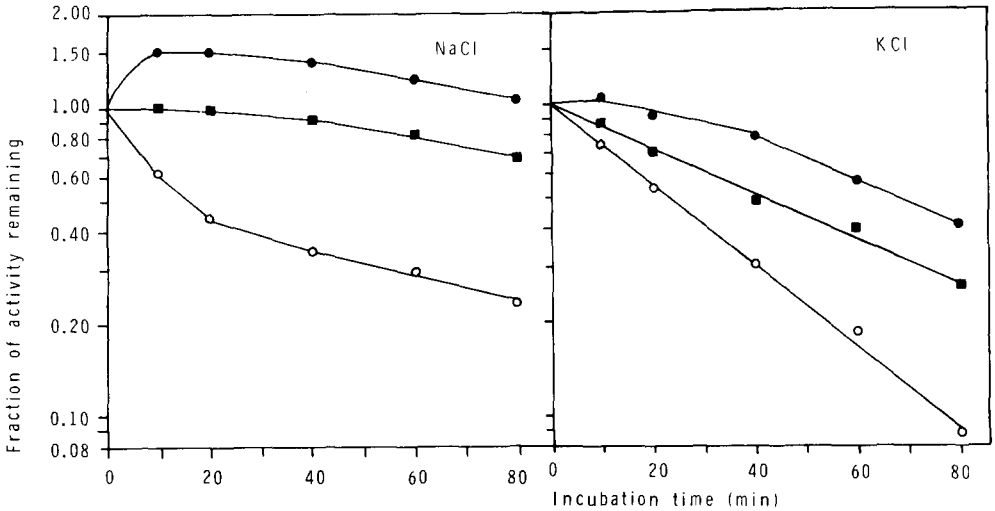

Fig. 7. First-order plots of tryptic modification of (•) Na^+-dependent (^{14}C)-ADP-ATP exchange, (▪) ($Na^+ + K^+$)-dependent (^{14}C)-ADP-ATP exchange, and (○) ($Na^+ + K^+$)-ATPase activities in presence of 150 mM NaCl (left) or 150 mM KCl (right). Incubation with trypsin and 150 mM NaCl, isolation of the enzyme and ($Na^+ + K^+$)-ATPase assay as in Fig. 3.

For assay of exchange activity, aliquots each containing about 5 μg protein were incubated for 15 or 30 min at 26°C in a total volume of 100 μl with 5 mM ATP, 1.25 mM [^{14}C]ADP, 5 mM $MgCl_2$ and 150 mM NaCl or 130 mM NaCl and 20 mM KCl. The nucleotides were separated by thin-layer chromatography on 20 cm polygram gel 300 polyethyleneimine-impregnated plastic sheets in 1.2 M LiCl. The spots were cut out with scissors and were eluted with 0.7 M $MgCl_2$, 25 mM Tris [19]. The radioactivity in ADP and ATP was determined in a Packard scintillation counter. The exchange activity in the control preparations was 61 nmol/min per mg protein with 150 mM NaCl and 142 nmol ATP formed/min per mg protein with 130 mM NaCl and 20 mM KCl.

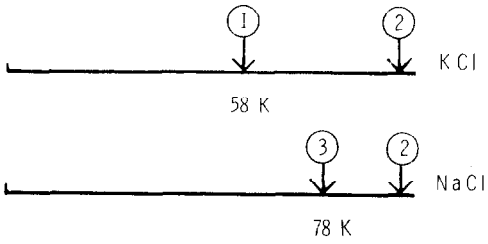

Fig. 8. Model of tryptic cleavage of the catalytic protein of $(Na^+ + K^+)$-ATPase in presence of NaCl or KCl. Bond 1 is cleaved with KCl in the medium producing the fragments with M_r = 58 000 and 46 000. The fragments arising by cleavage of bond 2 has not been identified; but the split is assumed to occur with both NaCl and KCl in the medium. With NaCl bond 2 is exposed and cleaved rapidly in phase A. With KCl bond 2 is protected and the split is preceded by cleavage of bond 1. Bond 3 is split in phase B with NaCl producing the fragment with M_r = 78 000.

reduced the $(Na^+ + K^+)$-ADP-ATP exchange activity (Fig. 8) at rates which were similar to or lower than the rates of reduction of ATP binding capacity (Fig. 3).

Discussion

The two distinct patterns of tryptic modification of the catalytic functions of $(Na^+ + K^+)$-ATPase seen with KCl and NaCl can be related to the previously described patterns of tryptic inactivation of $(Na^+ + K^+)$-ATPase and to the cleavage of the large catalytic protein [1]. There is no such relationship to cleavage of the smaller glycoprotein, which is much more resistant to proteolysis than the large chain [12].

With KCl, tryptic cleavage of the catalytic protein to almost equal fragments (Fig. 8, bond 1) abolishes ATP binding and phosphorylation in parallel to the inactivation of $(Na^+ + K^+)$-ATPase. Inactivation of the phosphatase activity seems to require an additional split (Fig. 8, bond 2).

With NaCl, there is a clear dissociation between modification of the various catalytic functions. In phase A, the rapid inactivation of 50—55% of the $(Na^+ + K^+)$-ATPase activity is associated with loss of 85% of the K^+-phosphatase activity and an increase of the Na^+-dependent ADP-ATP exchange activity to 150% of control. The cleavage responsible for this modification has not been identified, but may occur close to a terminal end of the catalytic protein (Fig. 8, bond 2). ATP binding and phosphorylation are not affected in phase A, but are lost slowly in phase B in parallel to cleavage of bond 3, Fig. 8.

The ATP binding area is more stable to trypsin with NaCl than with KCl, but the loss of binding capacity can in both cases be correlated to a distinct cleavage of the large chain, Fig. 8, bond 1 with KCl and bond 3 with NaCl. This is consistent with localization of the ATP binding area to the large chain and both bonds may form a part of this area if the protein is folded to bring the residues near to each other. It is, however, also possible that the loss of ATP binding can be secondary to a change in protein structure following cleavage of bonds remote from the binding area.

Contrary to the ATP binding site, the site for K^+-dependent hydrolysis of *p*-nitrophenyl phosphate is exposed to tryptic attack with NaCl in the medium and 85% of the phosphatase activity is lost in phase A in which ATP binding

is not affected. Also with KCl, inactivation of the phosphatase activity requires hydrolysis of a bond in addition to the split which abolishes ATP binding. These results suggest that the phosphatase site is located to a segment of the large chain which is distinct from that forming the ATP binding area.

Cleavage of bonds which are essential for binding of ATP (step a in the model for hydrolysis of ATP [1]) thus forms a good explanation for the inactivation of $(Na^+ + K^+)$-ATPase with KCl in the medium and in phase B with NaCl in the medium.

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

(a) (b) (c) (d)

The mechanism of inactivation of $(Na^+ + K^+)$-ATPase in phase A with NaCl must be entirely different since 50—55% of the activity is lost without apparent changes in ATP binding and Na^+-dependent phosphorylation (steps a, b and c). This inactivation may therefore result from cleavage of bonds involved in dephosphorylation reactions (step d). Interference with step d may also explain the reciprocal changes in the K^+-phosphatase and the Na^+-dependent ADP-ATP exchange activities.

This demonstration of changes in phosphatase and exchange activities without modification of ATP binding and phosphorylation levels is of considerable interest for future studies of the role of the phosphorylated intermediate. After partial tryptic digestion with NaCl to a $(Na^+ + K^+)$-ATPase activity of about 40% of control, a stable preparation can be isolated in which the cleavage in phase A has been completed, whereas the cleavage in phase B has barely begun. Its capacity for ATP binding and phosphorylation is close to 90%, the K^+-phosphatase activity is about 15% and the Na^+-dependent ADP-ATP exchange activity about 150% of control. It will be of interest to learn if these activity levels can be correlated to changes in phosphorylation kinetics.

The unit of $(Na^+ + K^+)$-ATPase which binds one molecule ATP or ouabain has a molecular weight of 270 000—280 000 and seems to operate as a dimer with each monomer containing one large chain and one small glycoprotein [7]. The monomer is seen on the membrane surface by electron microscopy after negative staining [13] and is solubilized by Triton as a protein complex with molecular weight 140 000 [14]. Independent evidence for this subunit structure has been obtained in cross-linking experiments [15,16]. The present results provide indirect evidence for localization of the ATP binding area to only one of the two large chains in the dimer because the data yield a linear relationship between the fractional loss of ATP binding capacity and cleavage of the large chain in the presence of KCl. With KCl, all large chains are cleaved at the same rate [1] and it is reasonable to assume that the cleavage is random. With this assumption a linear relationship is obtained only if the ATP binding area is located to one of the two large chains. If the two large chains in the dimer were identical, binding of ATP could either be a common function with equal participation of the two chains (case A) or ATP could bind to one of two identical chains and binding to one chain could exclude binding to the other by subunit interaction (case B). Either case would give a curvilienear relation-

ship between the loss of ATP binding and cleavage of the large chain. In case A there would be a rapid initial loss of ATP binding sites since binding would be abolished by cleavage of either of the two chains or of both chains in the dimer. In case B the initial loss of ATP binding sites would be much slower than the rate of cleavage since both chains should be cleaved to eliminate binding.

One possible explanation for the biphasic pattern of tryptic digestion of $(Na^+ + K^+)$-ATPase in the presence of NaCl is that it is due to cleavage at different rates of two distinct large chains. The hypothesis emerging from the experiments is therefore that the unit of $(Na^+ + K^+)$-ATPase which binds one molecule of ATP contains two large chains which are not identical. One chain contains the ATP binding area and the other chain has a site for hydrolysis of acyl phosphates. With this subunit structure, separation of two catalytic proteins with molecular weight close to 100 000 is required for adequate analysis of the protein structure of $(Na^+ + K^+)$-ATPase.

It has been proposed that channels for transport of ions across membranes are formed in clefts between the subunits of membrane protein complexes [17]. However, in the three-dimensional model proposed for the proton pump of Halobacteria [18] each protein unit forms a channel surrounded by 7 α-helices which extend through the membrane. It can be calculated that formation of 7 similar helical structures extending through a membrane with a thickness of 90 Å requires less than half the total number of amino acids in a protein with molecular weight of 100 000. It is therefore a reasonable extension of the above hypothesis to propose a model in which each of the two large chains in the dimer forms a cation channel. Since the two chains seem to have distinct catalytic functions, they may also provide separate channels for transport of Na^+ and K^+.

References

1 Jørgensen, P.L. (1975) Biochim. Biophys. Acta 401, 399—415
2 Giotta, G.J. (1975) J. Biol. Chem. 250, 5159—5164
3 Erdmann, E. and Schoner, W. (1973) Biochim. Biophys. Acta 330, 316—324
4 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36—52
5 Jørgensen, P.L. (1974) Methods Enzymol. 32, 277—290
6 Walsh, K.A. (1970) Methods Enzymol. 19, 41—63
7 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 53—67
8 Jørgensen, P.L. (1975) Q. Rev. Biophys. 7, 239—274
9 Norby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104—116
10 Ray, W.J. and Koshland, D.E. (1961) J. Biol. Chem. 236, 1973—1979
11 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 258—273
12 Churchill, L. and Hokin, L.E. (1976) Biochim. Biophys. Acta 434, 258—264
13 Maunsbach, A.B. and Jørgensen, P.L. (1974) Proceedings of the VIII International Congress on Electron Microscopy, Vol. 2, pp. 214—215
14 Clarke, S. (1975) J. Biol. Chem. 250, 5459—5469
15 Giotta, G.J. (1976) J. Biol. Chem. 251, 1247—1252
16 Kyte, J. (1975) J. Biol. Chem. 250, 7443—7449
17 Jardetzky, O. (1966) Nature 211, 969—970
18 Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28—32
19 Randerath, K. and Randerath, E. (1967) Methods Enzymol. 12A, 323—347