Biochimica et Biophysica Acta, 507 (1978) 8—16

BBA 77914

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

VII. TRYPTIC DEGRADATION OF THE Na-FORM OF THE ENZYME PROTEIN RESULTING IN SELECTIVE MODIFICATION OF DEPHOSPHORYLATION REACTIONS OF THE (Na^+ + K^+)-ATPase

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(Received June 23rd, 1977)

Summary

1. We have examined the effect of partial tryptic digestion of purified (Na^+ + K^+)-ATPase in the presence of NaCl on the formation and degradation of the phosphoenzyme formed from ATP. The digestion to a (Na^+ + K^+)-ATPase activity of 40% barely affects ATP binding and the steady-state level of phosphorylation, but increases the rate of the Na^+-ADP-ATP exchange to 150% of control and reduces the K^+-phosphatase activity to 15—20% of control (Jørgensen, P.L. (1977) Biochim. Biophys. Acta 466, 97—108).

2. After this tryptic cleavage in the presence of NaCl, the fraction of ADP sensitive phosphoenzyme was 2—3-fold higher than control levels. The presence of KCl depressed the phosphoenzyme level 2—3-fold less than that of the control and the K^+-dependent increment of the rate constant for dephosphorylation was less than half that of the control.

3. The results show that partial tryptic digestion of the purified (Na^+ + K^+)-ATPase in the presence of NaCl selectively affects protein areas involved both in the transformation of the phosphoenzyme from an ADP-sensitive to an ADP-insensitive form and in the stimulation by K^+ of the dephosphorylation process and the K^+-phosphatase activity. The protein areas that are involved in these reactions seem therefore to be close to one another, or identical, and they appear to be spatially separated from the ATP binding area.

Introduction

Graded trypsinolysis of the purified, membrane-bound (Na^+ + K^+)-ATPase provides a tool for identifying conformational states of the sodium pump protein [1] and for localizing catalytic sites [2] and intramembraneous segments

[16] within the catalytic protein. In the present work we have examined a selective impairment of dephosphorylation reactions which is induced by tryptic cleavage in the presence of NaCl.

Tryptic digestion of ($Na^+ + K^+$)-ATPase in the presence of NaCl is biphasic. In phase A, the rapid inactivation of 50% of the ($Na^+ + K^+$)-ATPase activity is associated with a loss of 80% of the K^+-phosphatase activity and an increase in the Na^+-ADP-ATP exchange activity to 150% of control. ATP binding and phosphorylation are not changed in phase A but are lost slowly in phase B in parallel to the inactivation of ($Na^+ + K^+$)-ATPase and to cleavage of the large chain to a fragment with M_r 78 000. Since the rate of digestion in phase A is 15—20-fold higher than in phase B, 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. The ($Na^+ + K^+$)-ATPase activity of this preparation is 40%, its capacity for ATP binding and phosphorylation is 80—90%, the K^+-phosphatase activity is 15% and the Na^+-ADP-ATP exchange activity is 150—160% of control [2].

The reciprocal changes in phosphatase and exchange activities with unchanged levels of ATP binding and phosphorylation suggest that the tryptic split affects dephosphorylation reactions. In the present study we have therefore examined the effect of tryptic digestion in phase A in the presence of NaCl on the levels of phosphorylation from [γ-^{32}P]ATP with and without KCl in the medium, on the fractions of ADP-sensitive and ADP-insensitive phosphoenzyme, and on the rates of spontaneous and K^+-stimulated dephosphorylation.

Experimental

($Na^+ + K^+$)-ATPase from the outer medulla of rabbit kidney was purified in membrane-bound form by incubation of a microsomal fraction with sodium dodecyl sulphate and ATP followed by an isopycnic-zonal centrifugation [3].

A reproducible inactivation of ($Na^+ + K^+$)-ATPase to 40% of control activity was obtained by incubation of 1 I.U. trypsin per 100 μg enzyme protein in 25 mM imidazole buffer with pH 7.5 and at 37°C for 20 min with 150 mM NaCl. Digestion was stopped by mixing with soyabean trypsin inhibitor (Sigma type 1-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 Beckman 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 for gel electrophoresis and assay of ($Na^+ + K^+$)-ATPase, ATP binding, K^+-nitrophenylphosphatase and ADP-ATP exchange activity as before [2]. For assay of ATPase activity at 2°C aliquots containing 30 μg protein were mixed with 149—150 mM NaCl/0—1 mM KCl/3 mM $MgCl_2$/30 μM [γ-^{32}P]ATP/30 mM Tris, pH 7.4 (2°C) in a total volume of 1 ml. After incubation for 10 min, the reaction was stopped with 100 μl of 50% trichloroaceticacid and the ^{32}P released from [γ-^{32}P]ATP was assayed by scintillation counting after solvent separation of phosphomolybdate from ATP [4].

For phosphorylation, portions containing 60—100 μg ($Na^+ + K^+$)-ATPase protein was incubated at 2°C with 30 μM [γ-^{32}P]ATP (0.3 μCi · ml^{-1}, purified Tris salt [5]) in 3 ml of 3 mM $MgCl_2$/30 mM Tris · HCl, pH 7.4 (2°C)/149—

150 mM NaCl/0—1 mM KCl, the sum of the cation concentration being always 150 mM. The reaction was stopped with 3 ml 8% ice-cold perchloric acid, and the protein was separated by centrifugation and washed four times with 5% trichloroacetic acid, 0.6 mM P_i, 0.6 mM ATP. The precipitate was dissolved in 0.25 ml 1 M NaOH with heating for 30 min at 60°C. Portions of 25—50 μl were used for proteir analysis by the Lowry method [6] and 100 μl were transferred to 10 ml of scintillator solution and counted with 100% efficiency in a Packard scintillation counter. The addition of ligands and correction for blank values are described in figure legends.

Results

The time courses of tryptic modification of the catalytic functions of the $(Na^+ + K^+)$-ATPase and the patterns of native and cleaved proteins after digestion in presence of NaCl or KCl were described before [1,2]. For the experiments in this work, we used purified preparations of $(Na^+ + K^+)$-ATPase which had been digested with trypsin in the presence of NaCl to a $(Na^+ + K^+)$-ATPase activity of 40% of the activity in the untreated control preparation.

In agreement with the previous results, the data in Table I show that after this digestion the K^+-phosphatase activity was only 20% of control, and that the Na^+-dependent ADP-ATP exchange activity had increased to 145% of control. It is also seen that the rate of ATP hydrolysis under the conditions of the phosphorylation experiments, i.e., at 2°C and with 150 mM NaCl or with 149 mM NaCl plus 1 mM KCl was reduced to the same extent by tryptic digestion as the $(Na^+ + K^+)$-ATPase activity at 37°C. The limited scatter of these values illustrates that procedure for partial tryptic inactivation of the purified enzyme was very reproducible. The electrophoretic patterns in Fig. 1 show that the

TABLE I

ENZYMATIC PROPERTIES OF THE PURIFIED $(Na^+ + K^+)$ATPase AND OF THE PREPARATION OBTAINED AFTER PARTIAL TRYPTIC DIGESTION OF THE PURIFIED ENZYME TO AN $(Na^+ + K^+)$-ATPase ACTIVITY OF 40% IN THE PRESENCE OF 150 mM NaCl

Procedures and assays are described under Experimental. The results obtained for a consecutive series of trypsinated preparations are given in percent of the simultaneously prepared control preparations which were incubated with NaCl 150 mM without trypsin.

	Control preparation	After digestion in presence of NaCl
	μmol · min^{-1} · mg^{-1} protein	Per cent of control
$(Na^+ + K^+)$-ATPase (37°C)	29 — 35	39 ± 2 (9)
K^+-Phosphatase (37°C)	3.7— 4.6	20 ± 1 (4)
[^{14}C]ADP-ATP exchange (26°C)	nmol · min^{-1} · mg^{-1} protein	
150 mM NaCl	39 — 72	145 ± 5 (6)
130 mM NaCl, 20 mM KCl	78 —139	101 ± 5 (6)
ATPase, 30 μM ATP (2°C)		
150 mM NaCl	7.4	35 (2)
149 mM NaCl, 1 mM KCl	15.5	45 (2)

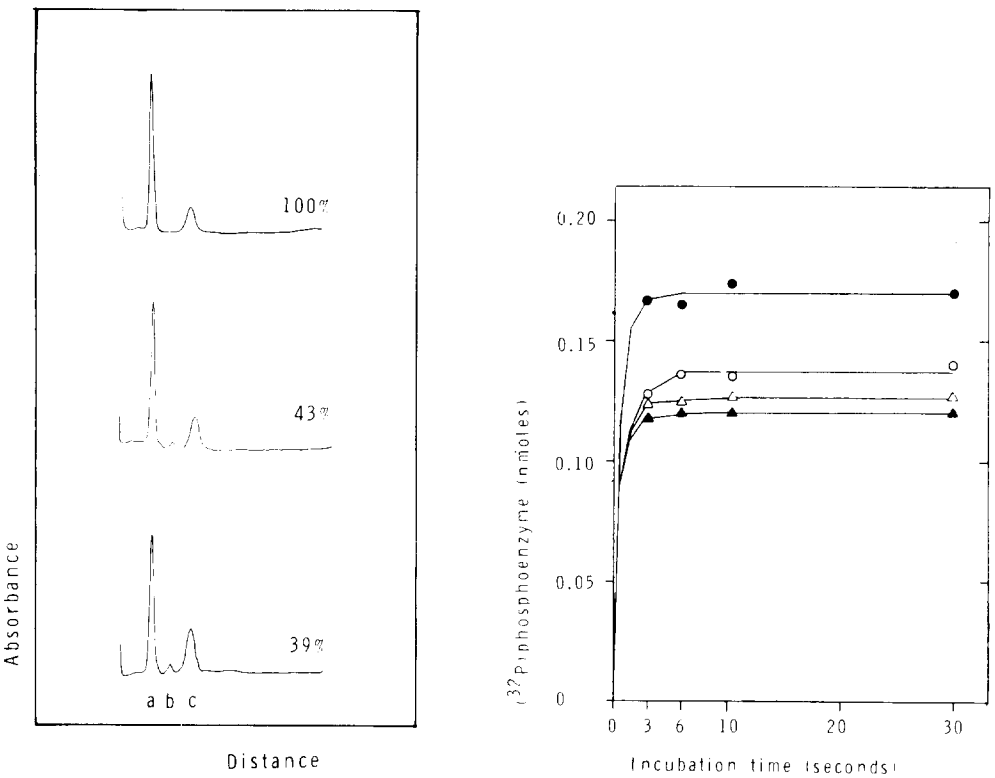

Fig. 1. Electrophoretograms of the purified $(Na^+ + K^+)$-ATPase and of the proteins remaining in the membranes after tryptic digestion to an $(Na^+ + K^+)$-ATPase activity of 43% and 39% the presence of 150 mM NaCl. The polyacrylamide gels were stained with Coomassie Blue and scanned as before [1]. Molecular weights of the bands were a, 98 000; b, 78 000; c, 58 000.

Fig. 2. Effect of tryptic digestion in the presence of NaCl on the steady-state levels of phosphoenzyme formed by $(Na^+ + K^+)$-ATPase. Aliquots containing 75 μg protein of trypsinated (△,○) and control preparations (▲,●) were phosphorylated at 2°C in presence of 3 mM $MgCl_2$, 150 mM NaCl, 30 μM [γ-^{32}P]ATP without (○,●) or with (△,▲) 200 μM KCl. As blank value the phosphorylation level with 150 mM KCl was subtracted.

digestion caused a very limited change in protein composition. The split responsible for the inactivation in phase A could not be identified and the appearance of a small amount of the peptide with molecular weight 78 000 could be related to the cleavage in phase B (cf. ref. 2).

In Fig. 2, the effect of K^+ on the steady-state levels of phosphoenzyme of the two preparations was examined. It is seen that the levels obtained after 3—6 s of incubation at 2°C remained stable for up to 30 s. The data in Fig. 2 are representative, as the average maximum level of Na^+-dependent phosphoenzyme for the seven trypsinated preparations, which were used in the present series of experiments, was 81 ± 4% (S.E., n = 7) of control. The absolute values for the control preparations fell in the range of 2.3—3.7 nmol · mg^{-1} protein.

Fig. 2 also shows that the presence of 200 μM KCl reduced the level of phosphoenzyme obtained with the trypsinated preparation much less (—8%) than the levels of phosphoenzyme obtained with the control (—21%).

This effect of K^+ on the steady-state level of phosphoenzyme was examined

over a wider range of K^+ concentrations in the experiment in Fig. 3. It is seen that the addition of K^+ reduced the steady-state phosphoenzyme level of the trypsinated preparation much less than that of the control.

This altered sensitivity of the phosphoenzyme level to potassium could suggest that dephosphorylation processes were impaired by the digestion, since potassium in the range of concentrations used in Figs. 2 and 3 is known to have its major effect on the dephosphorylation of the phosphoenzyme. Examination of the rates of spontaneous dephosphorylation after addition of excess unlabelled ATP [7] and of the K^+-stimulated dephosphorylation after addition of ATP plus KCl are shown in Figs. 4 and 5. Fig. 4 shows that the time course of dephosphorylation was linear during the first 6 s and that extrapolation of the values of zero time gave almost the same starting point, between 85% and 95% of the original amount of phosphoenzyme. This initial decrease in amount of phosphoenzyme may represent a rapid exchange of bound [γ-^{32}P]ATP with the unlabelled ATP added in excess at zero time [7]. It is seen from Fig. 4, that the rate of spontaneous dephosphorylation was nearly the same for the two preparations; whereas the K^+-stimulated increment of the rate constant for dephosphorylation was 2-fold larger for the control than for the trypsinated preparation.

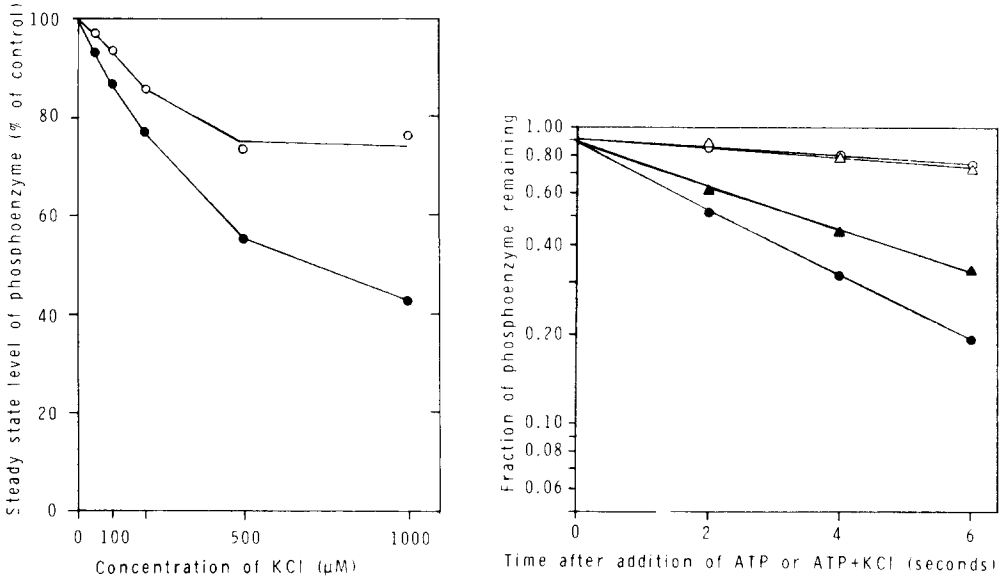

Fig. 3. Effect of K^+ on the steady-state levels of phosphoenzyme formed by the control preparation (●) and preparations which had been digested with trypsin (○) in the presence of NaCl. Incubation for 30 s as in Fig. 1 with 0—1000 μM KCl. As blank value the phosphorylation level with 150 mM KCl was subtracted.

Fig. 4. Effect of ATP or ATP plus 200 μM KCl on the rate of dephosphorylation of a control preparation (○,●) and a preparation that had been digested by trypsin in the presence of NaCl (△,▲). Aliquots containing about 100 μg protein were phosphorylated at 2°C in the presence of 30 μM [γ-^{32}P]ATP, 3 mM $MgCl_2$, 150 mM NaCl. After 6 s (zero time on the graph) 1 mM ATP (○,△) or 1 mM ATP plus 200 μM KCl (●,▲) were added and the amount of phosphoenzyme was determined 2, 4, and 6 s later. Zero time values were obtained by addition of perchloric acid before ATP or ATP plus KCl. As blank value was subtracted the phosphorylation level reached 2 min after addition of 1 mM ATP.

Fig. 5 shows the results of a series of determinations of dephosphorylation velocities as in Fig. 4, but using a wider range of K^+ concentrations. It is seen that addition at zero time of KCl in concentrations increasing up to 1 mM along with the unlabelled ATP gave a curvilinear increase in the rate constant determined from the linear dephosphorylation curves. The increase in rate constant for the control preparation was about 10-fold, from 0.047 ± 0.07 s^{-1} (S.E., n = 6) without KCl to 0.524 ± 0.004 s^{-1} (n = 3) with 1 mM KCl. With the trypsinated preparation, the increment was much smaller, about 6-fold, from 0.042 ± 0.007 s^{-1} (n = 5) to 0.28 s^{-1} (n = 2).

The calculated rate of hydrolysis which is the product of the rate constants of spontaneous dephosphorylation times the phosphorylation level ($EP \cdot k_d$) was close to the (Na^+)-ATPase activity at 2°C, for the control preparation; whereas for the trypsinated preparation, the product $EP \cdot k_d$, was 2—3-fold larger than the (Na^+)-ATPase activity at 2°C (Table I). This, taken in conjunction with the increase in (Na^+)-ADP-ATP exchange activity to 145% of control (Table I) suggested that the ADP-sensitivity of the phosphoenzyme formed by trypsinated preparation could be altered.

The reactivity to ADP of the phosphoenzymes formed in the presence of Na^+ was examined as in Fig. 6. It is seen that the time course of dephosphorylation after addition of ADP was biphasic. In the first phase, the disappearance of a part of the phosphoenzyme was so rapid that measurement of the velocity was impossible. The rate of dephosphorylation in the second phase was equal

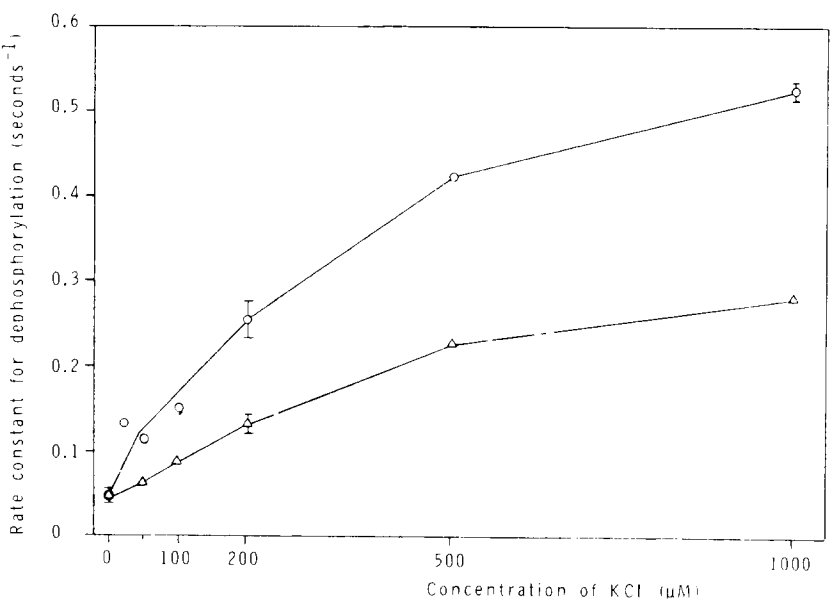

Fig. 5. Effect of KCl on the rate of dephosphorylation of control preparations (○) and of preparations that had been digested with trypsin in the presence of NaCl (△). Incubation as in Fig. 3. After 6 s, 1 mM ATP plus 0—1000 μM KCl were added and the amount of phosphoenzyme was determined 4 s later. Zero time values were obtained by adding perchloric acid before ATP plus KCl. The rate constants were calculated from the ratio of phosphoenzyme remaining after 4 s to 90% of the zero time phosphoenzyme level. Blank value as in Fig. 4.

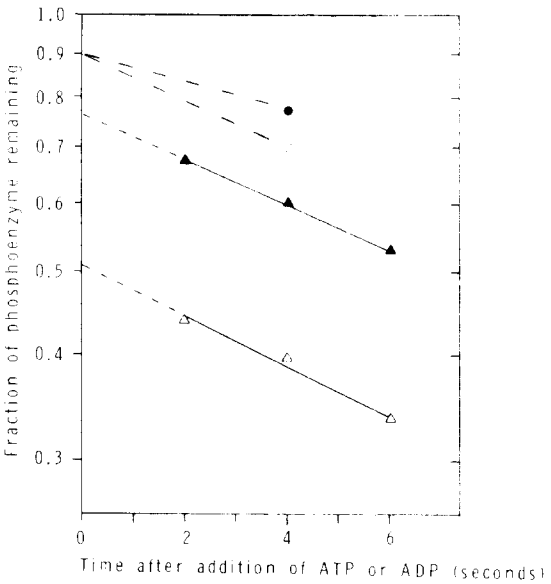

Fig. 6. ADP-dependent dephosphorylation of control preparations (▲) and of preparations that had been digested with trypsin (△) in presence of NaCl. After phosphorylation for 6 s as in Fig. 3 either 1 mM ATP (○,●) or 2,5 mM ADP (△,▲) were added and the amount of phosphoenzyme was determined after 2, 4, and 6 s. Zero time values were determined by addition of perchloric acid before ATP or ADP. Blank value as in Fig. 4.

to the rate of spontaneous dephosphorylation after ATP (Figs. 4 and 5) and it was nearly identical for the two preparations. This means that the velocity of transition from ADP-insensitive to ADP-sensitive phosphoenzyme forms was low enough to allow measurement of the fraction of ADP-sensitive phosphoenzyme under steady-state conditions by extrapolation of the second phase velocity to zero time (Fig. 6). After correction for ATP-exchangeable ^{32}P, the ADP-sensitive fraction formed 14% of the total amount of phosphoenzyme for the control and 40% for the trypsinated preparation. The experiment thus shows that tryptic digestion in phase A results in a 2—3-fold increase of the relative amount of ADP-sensitive phosphoenzyme.

Discussion

The tryptic digestion in phase A with NaCl in the medium is shown to alter the properties of the phosphoprotein which is formed from [γ-^{32}P]ATP in the presence of Na^+ and Mg^{2+}. The maximum concentration of phosphoenzyme is close to normal, but the fraction of ADP-sensitive phosphoenzyme is trebled and there is an abnormal reaction to addition of K^+. It is expressed in a weaker effect of K^+ on the steady-state level of phosphoenzyme and in a decrease in the velocity of the K^+ stimulated dephosphorylation. The binding capacity for ATP of this enzyme is 80—90% of control and the dissociation constant of the enzyme-ATP complex is not altered [2]. The catalytic defect is, therefore, selective and it is remarkable that it is caused by a very limited damage to the protein of the purified (Na^+ + K^+)-ATPase.

The combination of catalytic functions seen after tryptic digestion in phase A in the presence of NaCl appears to be unique. An increase in the fraction of ADP-sensitive phosphoenzyme and in the ADP-ATP exchange activity in combination with a decrease in the rate of dephosphorylation after stimulation with K^+ have been observed after poisoning with *N*-ethylmaleimide [8,9] and oligomycin [9], but these compounds do not inhibit the K^+-phosphatase activity more than the $(Na^+ + K^+)$-ATPase activity [10,11].

Quercitin [12] acts similarly, but it is not known whether it affects the K^+-phosphatase activity.

The increase in ratio of ADP-sensitive to ADP-insensitive phosphoenzyme seems to explain the increase in (Na^+)-ADP-ATP exchange activity. The number of ATP molecules formed per molecule of ADP-sensitive phosphoenzyme per unit time is nearly the same for the control and the trypsinated preparation. This means that the higher exchange activity after the digestion in the presence of NaCl is based on a larger number of phosphoenzyme units which produce ATP after addition of ADP rather than on a higher exchange velocity per enzyme unit.

The simultaneous loss of the K^+-phosphatase activity and the appearance of a dephosphorylation defect is of particular interest in view of the suggestion that the phosphatase activity reflects a K^+-dependent hydrolytic step of the overall ATP hydrolysis by $(Na^+ + K^+)$-ATPase (for reference see ref. 14). In support of this, the results show that the split which reduces the K^+-phosphatase activity also affects the sensitivity of the phosphoenzyme to K^+ and the distribution among ADP-sensitive and ADP-insensitive phosphoenzyme forms. On the other hand, the relationship is not quantitative, since the K^+-phosphatase activity after the digestion is 5-fold lower than that of the control, whereas the decrease in rate constant of K^+-sensitive dephosphorylation is only about 2-fold. The discrepancy does not preclude that the phosphatase activity reflects a dephosphorylation step, but may only reflect that *p*-nitrophenylphosphate is not an ideal substrate for the reaction.

The tryptic cleavage in phase A is not detectable by gel electrophoresis in sodium dodecylsulfate [2] but the split might be close to a terminal end of the catalytic protein because 10—20 amino acids may be removed from the large chain without altering significantly the electrophoretic mobility. This relatively modest change does not alter the properties of the ATP binding area or the part of the protein that is involved in formation of the ADP-sensitive phosphoenzyme, but is impairs the transition to the ADP-insensitive phosphoenzyme and the K^+-stimulated dephosphorylation and phosphatase reactions. This selective modification suggests that the protein areas that are involved in the dephosphorylation reactions, and in the phosphatase activity, are close to one another or even identical. These areas are exposed to trypsin in the presence of NaCl, when the catalytic protein is on the (t)Na-form and the ATP binding area is protected from tryptic attack. The transition to the (t)K-form of the catalytic protein following the exchange of NaCl for KCl is accompanied by exposure of the ATP binding area to trypsin and by protection of the phosphatase area [1,2]. Furthermore, the loss of the binding capacity for ATP can be correlated to distinct splits within the large chain, whereas the cleavage in phase A in the presence of NaCl is not detectable by gel electrophoresis [2].

These observations are compatible with the proposal that the protein areas which are engaged in the dephosphorylation and the phosphatase reactions are spatially separated from the ATP binding area.

Binding of ATP in the presence of NaCl does not alter the conformation of the catalytic protein, whereas the transfer of γ-phosphate from ATP to an aspartyl residue [14,15] is accompanied by transition from the (t)Na-form to the (t)K-form [1]. In view of the observations discussed above it is reasonable to propose that this change in protein structure involves a reduction in distance between the ATP binding area and the area responsible for the K^+-stimulated dephosphorylation reaction. At present it is not possible to tell whether the tryptic cleavage in phase A interferes with such a structural change or if the catalytic modifications are due to a damage inflicted on areas that are responsible for the binding of K^+ or for the hydrolysis of the aspartylphosphate.

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