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Conformational states of ($K^+ + H^+$)-ATPase studied using tryptic digestion as a tool

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The ($K^+ + H^+$)-ATPase from gastric mucosa has been treated by limited proteolytic digestion with trypsin to study the conformational states of the enzyme. The existence of a K^+- and an ATP-form of the enzyme follows from the kinetics of inactivation and from the specific cleavage products. In the presence of K^+ the 95 kDa chain is cleaved into two fragments of 56 and 42 kDa, whereas in the presence of ATP fragments of 67 and 35 kDa are formed. When Mg^{2+} is present during tryptic digestion cleavage products which are specific for both the ATP- and the K^+-form of the enzyme are yielded. In analogy to ATP, Mg^{2+} is able to convert the enzyme from a K^+-conformation to a more protected form. Moreover Mg^{2+} supports the protecting effect of ATP against tryptic inactivation. The $K_{0.5}$ for ATP is lowered from 1.6 mM (no Mg^{2+}) to 0.2 mM in the presence of 10 mM Mg^{2+}. Mg^{2+}, which in previous studies has been shown to induce a specific conformation, apparently induces a conformation different from the K^+-form of the enzyme and has ATP-like effects on the enzyme. In addition it has been found that in the initial rapid phase of the digestion process the K^+-ATPase activity is interrupted at a step which is very likely the interconversion of the phosphoenzyme forms E_1P and E_2P, since neither the K^+-stimulated *p*-nitrophenylphosphatase activity nor the phosphorylation of the enzyme are inhibited in this phase. During the tryptic digestion in the presence of K^+ there is a good correlation between the residual ATPase activity and the amount of the catalytic subunit left, suggesting that the latter is homogeneous. After tryptic digestion in the presence of K^+, phosphorylation only occurs in the 42 kDa and not in the 56 kDa band. The same experiments in the presence of ATP yield only phosphorylation in the 67 kDa band and not in the 35 kDa band. A provisional model for the structure of the catalytic subunit is given.

Introduction

The enzyme ($K^+ + H^+$)-ATPase (EC 3.6.1.36) derived from the gastric parietal cells is involved in gastric acid secretion and has been shown to catalyze an electroneutral exchange of H^+ for K^+ ions [1–4]. The enzyme is an oligomer of several subunits of the same molecular mass of approx. 100 kDa. The homogeneity of these subunits has been disputed [5–7]. Recently the molecular mass of rat gastric ($K^+ + H^+$)-ATPase has been established to be 114012 Da [8].

From studies with chemical modifying agents and fluorescent probes at least three conformational states of the enzyme have been identified: an ATP-conformation, a Mg^{2+}-form and a K^+-form [9–14]. However, these studies do not provide detailed molecular information on these con-

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formational states. Moreover it would be of interest to have more information on how these conformational states are interconverted and how the affinity of binding of ligands changes concurrently.

An approach which offers the possibility to get this information is limited proteolytic digestion of the $(K^+ + H^+)$-ATPase in combination with polyacrylamide gel electrophoresis in SDS. With Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase [15–18] studies on digestion with proteolytic enzymes have thus far been informative on the structure of the catalytic subunit of these enzymes and will even more be so in combination with the recently obtained knowledge on the primary structure of the catalytic subunit of these enzymes [18,19,8].

Saccomani et al. [5] have reported on trypsin digestion of the $(K^+ + H^+)$-ATPase. A specific protection by ATP and ADP resulting in a distinct peptide pattern has been observed. Relative to the situation when no extra ions are present, Na^+ and K^+ at high concentrations reduced the rate of loss of activity but no change in the peptide pattern was observed. From these studies they concluded moreover that the main protein subunit of $(K^+ + H^+)$-ATPase is heterogeneous, which conclusion has been challenged by Peters et al. [6].

The effect of Mg^{2+} ions on tryptic inactivation of $(K^+ + H^+)$-ATPase has not been studied yet. In this study we have made this investigation and compared the arising fragments with those yielded in the presence of K^+, ATP and Na^+.

Materials and Methods

Isolation of $(K^+ + H^+)$-ATPase. $(K^+ + H^+)$-ATPase was purified from gastric fundic mucosa as described before [20]. The specific activity of the preparations ranges from 70 to 110 μmol per mg protein per hour. The steady-state level of phosphorylation with 5 μM ATP ranges from 1.0 to 1.4 nmol incorporation per mg protein.

Tryptic degradation of the enzyme. Treatment with trypsin was carried out at 37 °C in 25 mM imidazole-HCl (pH 7.5) and at 1.5 mg/ml $(K^+ + H^+)$-ATPase. Trypsin and membrane protein were used in weight ratios of approx. 1 : 100, the exact value being dependent on the cations (Table I). Both trypsin and $(K^+ + H^+)$-ATPase suspension were incubated separately at 37 °C prior to mixing. Digestion was started by addition of trypsin. At selected time intervals aliquots were taken and added to trypsin inhibitor in a weight ratio of trypsin to inhibitor of 1 : 10. From the treated samples aliquots were taken for enzyme assays and polyacrylamide gel electrophoresis (PAGE) in SDS. In studies on the time course of inactivation of $(K^+ + H^+)$-ATPase by trypsin, the activities were calculated as fraction of the original activities remaining and were plotted against time in semi-logarithmic plots. Zero time activities were determined before the addition of trypsin.

Control experiments. The tryptic activity was tested at 37 °C in 1 ml medium containing 25 mM imidazole-HCl (pH 7.5), 0.5 mM *N*-α-benzoylarginine-L-*p*-nitroanilide (BAPNA, purchased from Sigma) and various ligands. After 10 min incubation at 37 °C, the reaction was stopped by addition of 1 ml ice-cold trypsin inhibitor in a weight ratio of inhibitor to trypsin of 10 : 1. The enzymatic activity of trypsin was measured by

TABLE I

THE ACTIVITY OF TRYPSIN UNDER VARIOUS CIRCUMSTANCES AS DETERMINED WITH AN ARTIFICIAL SUBSTRATE

Tryptic activity was tested at 37 °C in 1 ml medium containing 25 mM imidazole-HCl (pH 7.5), 0.5 mM *N*-α-benzoylarginine-L-*p*-nitroanilide (Sigma) and the indicated concentrations of ligands and trypsin. After 10 min incubation at 37 °C the reaction is stopped by addition of 1 ml ice-cold trypsin inhibitor in a trypsin: inhibitor ratio of 1 : 10. The enzymatic activity is given as the absorbance of *p*-nitroanilide per min. Values of two experiments are given. The trypsin concentrations indicated in the table are used in further experiments.

Additions	Trypsin concn. (μg/ml)	Activity (A_{405}/min)	
–	17	35.6	39.3
100 mM choline-Cl	15	36.8	40.4
30 mM $MgCl_2$	10	38.3	40.0
30 mM $CaCl_2$	10	40.0	39.8
100 mM KCl	16	37.9	40.6
100 mM NaCl	12	37.7	40.8
5 mM ATP, 90 mM choline-Cl	25	35.2	39.0
5 mM ADP, 90 mM choline-Cl	28	35.5	39.8
1 mM EDTA, 100 mM choline-Cl	28	40.0	40.5

monitoring the absorbance of *p*-nitroanilide at 405 nm.

Gel electrophoresis. For analysis of the samples by polyacrylamide-gel electrophoresis (PAGE) in SDS (sodium dodecylsulphate), aliquots of trypsinized protein were added to 500 µl 5% trichloroacetic acid at 0 °C. After 2 min centrifugation at 16000 rpm, the pellets were washed with 300 µl 30% (w/v) sucrose. The pellets were resuspended in sample buffer containing 50 mM Tris-HCl (pH 6.8), 2.5% dithioerythritol, 2% SDS, 10% glycerol and 0.006% Bromophenol blue. Solubilization was overnight at room temperature. Samples of 30 µl, containing 25 µg protein, were applied to slab gels. Gradient gels were prepared with a polyacrylamide concentration of 8–15% (w/v) and run according to Laemmli [21]. Molecular weight standards were: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20.1 kDa) (Pharmacia). Electrophoresis was performed at 20 °C for 5–6 hours at a current of 20 mA. The gels were stained with Coomassie brilliant blue and scanned with a LKB-Bromma 2202 Ultra Densitometer in combination with a LKB Bromma recording integrator. The presence of a 20 kDa band in some gels is apparently due to trypsin inhibitor not sufficiently removed from $(K^+ + H^+)$-ATPase at the centrifugation step.

ATPase and p-nitrophenylphosphatase assay. K^+-ATPase activity and K^+-stimulated *p*-nitrophenylphosphatase activity of the enzyme was measured in 30 mM imidazole-HCl (pH 7.5), 5 mM Mg^{2+}, 0.1 mM ouabain, 20 mM KCl or 20 mM choline chloride and either 5 mM Na_2ATP or 5 mM *p*-nitrophenyl phosphate. To 400 µl of this medium 20 µl enzyme suspension was added after which incubation took place for 10–30 min at 37 °C. Stopping of the reaction and further treatment of the samples was carried out as described before [22]. The K^+-ATPase and K^+-phosphatase activity was obtained form the difference in activity with KCl and choline chloride, respectively. Protein was determined according to Lowry et al. [23], using bovine serum albumin as standard.

Phosphorylation of $(K^+ + H^+)$-ATPase samples. For phosphorylation of the enzyme after trypsin treatment, samples were prepared as described above. After addition of trypsin inhibitor samples of 150 µl were applied to a Sephadex G-25 (coarse) in order to remove ATP and cations. A 10 cm column of 5 mm diameter was used, equilibrated with 25 mM imidazole-HCl (pH 7.5). After applying the enzyme sample the column was eluted with a total volume of 1 ml of the before mentioned buffer. The last 500 µl buffer eluted was collected. The protein content of the collected samples was determined according to Lowry et al. [23], using bovine serum albumin as a standard.

For phosphorylation aliquots of 30 µg protein were incubated in 150 µl medium containing 25 mM imidazole-HCl (pH 7.0), 1 mM Mg^{2+} and 20 µM [γ-^{32}P]ATP (final concentrations). After 15 s at 30 °C phosphorylation was stopped by addition of a ml 5% trichloroacetic acid (w/v), 0.1 M H_3PO_4. Incorporation of [^{32}P]P_i was determined as described before [20]. For analysis of the ^{32}P-labeled enzyme peptides by SDS-polyacrylamide gel-electrophoresis the samples were treated as described above. Solubilization was 30 min at 37 °C and gel electrophoresis was performed at 0 °C. After completion of the gel electrophoresis the gel was cut in 2 mm slices, which were screened for their ^{32}P-content in 5 ml scintillation fluid.

Although the phosphoenzyme is rather labile at the used pH and at 37 °C, we preferred to use this type of gelelectrophoresis as it gave the best resolution of peptide bands. The amount of phosphoenzyme left after gel electrophoresis is estimated by us to be 10% of the original level.

Results

A controlled rate of inactivation of $(K^+ + H^+)$-ATPase by trypsin has been obtained by adjusting the ratio of trypsin to enzyme protein. As observed in experiments on tryptic inactivation of $(Na^+ + K^+)$-ATPase [17] and of $(K^+ + H^+)$-ATPase [5], salts counteract the tryptic inactivation process. It was therefore important to keep the ionic strength constant in examination of the effects of ions and nucleotides on the digestion process. The inactivation of $(K^+ + H^+)$-ATPase was stopped completely by addition of trypsin inhibitor when the weight ratio of inhibitor to trypsin was 5 or higher.

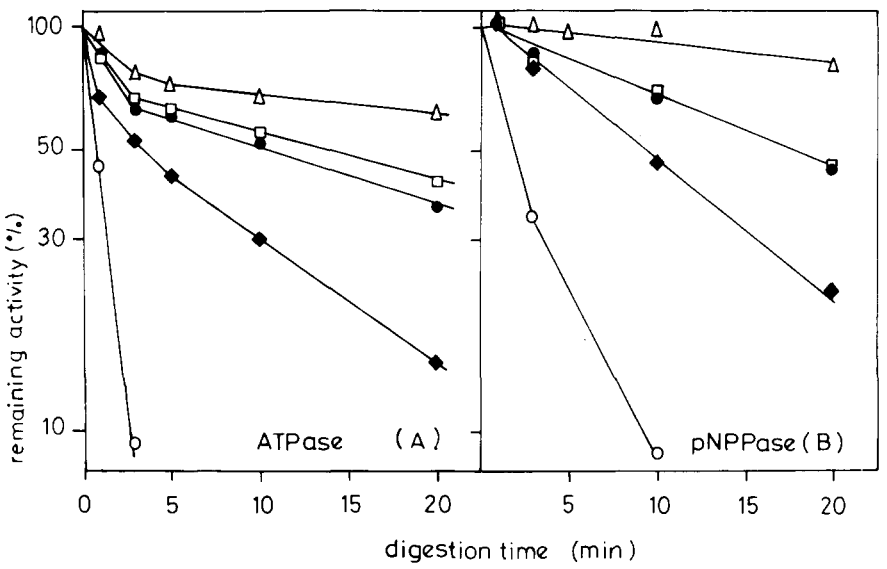


Fig. 1. Inactivation of $(K^+ + H^+)$-ATPase activity by trypsin in the presence of various ligands. Aliquots of $(K^+ + H^+)$-ATPase preparation, containing 150 μg protein were incubated at 37 °C in 100 μl medium, containing 30 mM imidazole-HCl (pH 7.5) with no ligands (○———○) or the ligands: 10 mM ATP plus 100 mM choline chloride (△———△), 100 mM KCl (◆———◆), 30 mM $MgCl_2$ (□———□) or 100 mM NaCl (●———●). Trypsin concentrations varied from 10 to 29 μg/ml as indicated in Table I. At the indicated intervals 24 μl of the incubation mixture was added to an ice-cold trypsin inhibitor solution (12 μl containing 5 μg trypsin inhibitor) and stored in ice. Aliquots containing 4.5 μg were used for determination of the K^+-stimulated ATPase activity (A) or K^+-stimulated *p*-nitrophenylphosphatase (K^+-phosphatase) (B) as described in Materials and Methods.

Table I shows the activity of trypsin with an artificial substrate in relation to the presence of various ligands. It is shown that divalent cations (Mg^{2+} and Ca^{2+}) stimulate the trypsin activity. ATP inhibits trypsin activity and EDTA has the same effect. This may be due to chelation of activating (divalent) cations present in the trypsin preparation. K^+ and choline ions have only a slight effect on the activity of trypsin. The trypsin concentrations indicated in Table I have been used in the further experiments on digestion of $(K^+ + H^+)$-ATPase.

Effects of various ligands on the digestion of $(K^+ + H^+)$-ATPase

The semilogarithmic plots of Fig. 1A show that inactivation of K^+stimulated ATPase activity is counteracted by high ionic strength. In the presence of K^+, as fast bi-exponential process is observed. The rate constant for inactivation in the slow phase is 0.073 min^{-1} (S.E. = 0.02, $n = 4$), while the fast phase is at least 20-times faster.

In the presence of ATP inactivation of $(K^+ + H^+)$-ATPase is very slow. A relatively rapid initial phase is observed with a rate constant of 0.08 min^{-1} (S.E. = 0.01, $n = 2$) and a very slow phase with a rate constant of 0.01 min^{-1} (SE = 0.002, $n = 4$). In the presence of Mg^{2+} and Na^+ the inactivation curve is intermediate between the K^+ and the ATP curve and is also bi-exponential with rate constants for the fast phase of 0.18 min^{-1} and 0.16 min^{-1} for Na^+ and Mg^{2+}, respectively, and of 0.03 min^{-1} for the slow phase for both.

Tryptic inactivation of the K^+-phosphatase activity shows quite different time dependence (Fig. 1B). The semilogarithmic plot of inactivation shows an initial phase (approx. 1 min) in which no inactivation but even stimulation (max. 10%) of the K^+-stimulated *p*-nitrophenylphosphatase activity is observed. A simple explanation of this phenomenon would be insufficient initiation of the tryptic degradation process. However, this is not the case as the experiments of Fig. 1B have been performed in parallel to the experiments of Fig. 1A which show a rapid initial phase of inactivation. After the first minute the time course

of inactivation of *p*-nitrophenylphosphatase activity is approximately linear on a semilogarithmic scale and rate constants are measured of 0.076 min^{-1} in the presence of K^+, 0.0011 min^{-1} with ATP and 0.0038 min^{-1} with Na^+ or Mg^{2+} in the medium.

Correlation of tryptic inactivation of K^+-ATPase, K^+-phosphatase and phosphorylation capacity

Fig. 2 shows the correlation between the time course of inactivation of the K^+-ATPase activity, K^+-phosphatase activity and the phosphorylation capacity of one enzyme preparation, digested by trypsin in the presence of 100 mM KCl. At the indicated time intervals samples were taken from the incubation mixture for determination of the mentioned activities. This confirms the discrepancy between inactivation of ATPase and phosphatase activity as already observed in Fig. 1. Moreover it is shown that the phosphorylation capacity and phosphatase activity have the same time course of inactivation. Similar results have been obtained in experiments on digestion in the presence of Na^+, Mg^{2+} or ATP.

Effect on protein composition

The electrophoretic pattern of the $(K^+ + H^+)$-ATPase preparation after trypsin digestion (Figs. 3 and 4) shows that the control, which is not incubated with trypsin, has a major protein band with an apparent molecular mass of 95 kDa. After

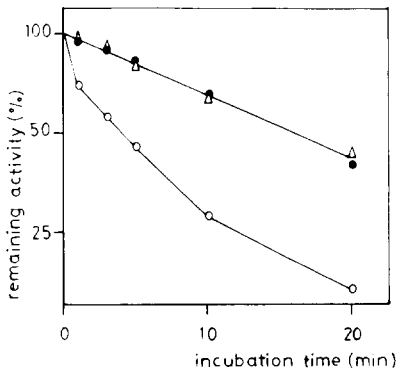


Fig. 2. Inactivation of $(K^+ + H^+)$-ATPase partial reactions by trypsin in the presence of KCl. The enzyme was treated as described in the legend to Fig. 1. Aliquots of 6 μg $(K^+ + H^+)$-ATPase were used for determination of K^+-stimulated ATPase (○——○), K^+-phosphatase (●——●) or phosphorylation from [γ-^{32}P]ATP (△——△) as described in Materials and Methods. The 100% level of phosphorylation refers to 1280 pmol per mg.

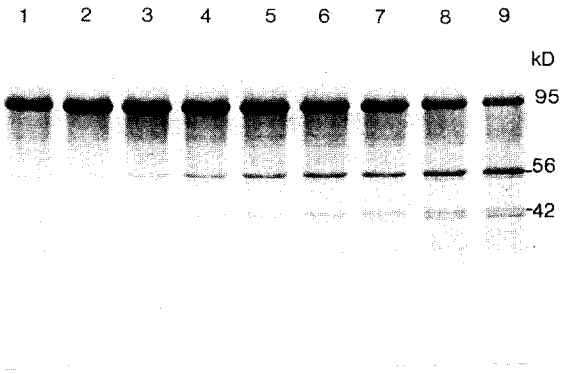


Fig. 3. Electrophoretic pattern of $(K^+ + H^+)$-ATPase peptides after tryptic digestion in the presence of KCl. $(K^+ + H^+)$-ATPase was incubated in the presence of 8 μg trypsin per ml and 100 mM KCl. Further treatment was as described in the legend to Fig. 2. The incubation time of the samples in the slots 1–9 was 0, 0.5, 1, 2, 3, 4, 5, 10 and 20 min, leaving an enzyme activity of 100, 96, 85, 77, 68, 68, 58 and 43% of the control activity (91 μmol/mg per h).

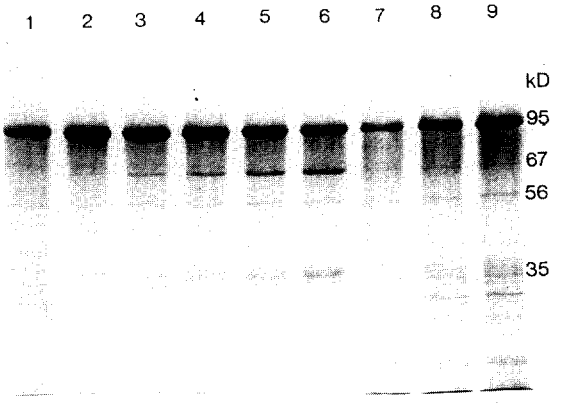


Fig. 4. Electrophoretic pattern of $(K^+ + H^+)$-ATPase peptides after trypsin digestion in the presence of ATP and Mg^{2+}. $(K^+ + H^+)$-ATPase was incubated in the presence of 5 mM ATP plus 1 mM EDTA and 90 mM choline chloride in the presence of 28 μg trypsin per ml for 1, 3, 5, 10 and 15 min, leaving 99, 77, 72, 68 and 64% of the control activity (slot 2–6). Slot 1 shows a control preparation that has not been incubated with trypsin. Slot 7–9 show quantities of $(K^+ + H^+)$-ATPase preparation (20, 30, 40 μg) digested in the presence of 30 mM Mg^{2+} as described in the legend to Fig. 1.

trypsin digestion with K^+ in the medium (Fig. 3) the pattern of peptides is distinctly different from that obtained with ATP in the medium (Fig. 4, lane 1–6). In the presence of K^+ the 95 kDa chain is cleaved into two fragments of 56 and 42 kDa, while later in process fragments occur with molecular masses of 40 kDa, 30 kDa and lower. The 56 kDa peptide is in some preparations accompanied by a fragment of 53 kDa. In the presence of ATP (with or without EDTA) a completely different peptide pattern is observed (Fig. 4). Two peptides are formed with apparent molecular masses of 67 and 35 kDa, respectively, which are most clearly present when the ATPase activity is diminished to approx. 50%. Even when digestion in the presence of ATP is prolongued no peptide pattern like in Fig. 3 is obtained.

When Mg^{2+} is present in the digestion medium the peptide pattern obtained is not as distinct as in the presence of K^+ or ATP (Fig. 4, lane 7–9). It appears that the peptides observed after digestion in the presence of Mg^{2+} are a combination of the peptides obtained with ATP or K^+ in the medium. The peptides obtained under 'Mg^{2+}'-conditionsare not very prominent as the experiment of Fig. 4 shows in which we have chosen the optimal point for showing the specific peptides. From this experiment it must be concluded that Mg^{2+} induces a conformation which is neither a specific K^+ nor a specific ATP conformation. Probably due to the fact that both K^+ and ATP splitting occurs with Mg^{2+} present, peptides are not accumulated to such an extent that prominent peptide bands occur.

In the first phase of the digestion process peptides occur with apparent molecular masses of 72 and 78 kDa. They are present after digestion in the presence of K^+, ATP and Mg^{2+} and probably also under other circumstances. These peptides are not very prominent, suggesting that they are rapidly cleaved into other peptides so their turnover is very fast. Hence no accumulation occurs as is the case with the other peptides.

Interaction of Mg^{2+}, ATP and K^+ in tryptic digestion

Fig. 5 shows the fraction of K^+-ATPase activity remaining after tryptic digestion for 15 min as a function of the ATP concentration in the absence

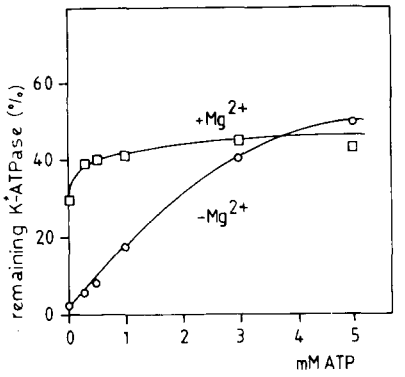


Fig. 5. Effect of $MgCl_2$ on the protective action of ATP against tryptic inactivation. $(K^+ + H^+)$-ATPase (1.5 mg/ml) was digested with trypsin (12 μg/ml) in the presence of ATP (0 to 5 mM). K^+-ATPase remaining after 15 min digestion with trypsin is given where (○——○) represents incubation without Mg^{2+} and (□——□) incubation in the presence of 10 mM Mg^{2+} in addition to ATP. Mixing with trypsin inhibitor and assay of K^+-ATPase activity were as described in the legend to Fig. 1.

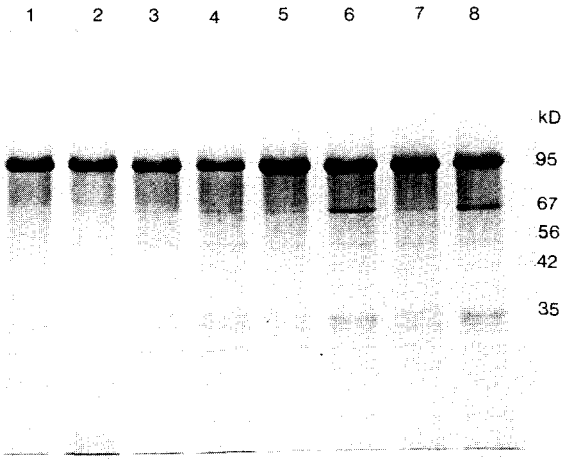


Fig. 6. Electrophoretic pattern of peptides obtained after digestion of purified $(K^+ + H^+)$-ATPase in the presence of Mg^{2+}, ATP and Mg^{2+} plus ATP. The enzyme (1.5 mg/ml) was incubated for 5 min (slot, 1, 3, 5, 7) or 20 min (slot 2, 4, 6, 8) in the absence (1, 2) or presence of trypsin (3–8). The trypsin concentration was 12 μg/ml (3, 4) or 16 μg/ml (5–8). Incubation occurred in the presence of 30 mM $MgCl_2$ (3, 4); 5 mM ATP, 1 mM EDTA plus 90 mM choline chloride (5, 6); 5 mM ATP plus 30 mM $MgCl_2$ (7, 8). Preparation of the samples and running of the gel was as described in Materials and Methods.

or presence of 10 mM Mg^{2+}. It is shown that beside the protective effect Mg^{2+} has of its own, it also enlarges the protecting effect of ATP against inactivation. In the absence of Mg^{2+}, the $K_{0.5}$ for ATP is 1.6 mM whereas in the presence of 10 mM Mg^{2+}, the $K_{0.5}$ for ATP is 0.2 mM (S.E. = 0.04, $n = 3$). The interpretation of this experiments is complicated by the fact that the trypsin concentration was constant during these experiments. It might be that part of the protecting effect of trypsin is due to inhibition of the trypsin activity. The latter effect is, however, the same with and without Mg^{2+}.

Fig. 6 shows that the peptide pattern obtained after digestion of $(K^+ + H^+)$-ATPase in the presence of ATP plus Mg^{2+} is the same as the pattern obtained with ATP plus EDTA. As in the presence of Mg^{2+} and ATP together the enzyme is phosphorylated, it must be concluded that with this method the conformation of the enzyme with ATP merely bound (E · ATP) which is assumed to prevail in the presence of EDTA [22,24] and the phosphorylated state (EP) cannot be discriminated.

The experiment given in Fig. 7 shows that the inactivation by trypsin in the presence of K^+ is antagonized by addition of ATP. At the highest ATP concentration used, the inactivation curve is approximately the same as with ATP alone. A half-effective concentration of approx. 2 mM ATP is calculated in the presence of 100 mM K^+. ATP presumably induces the enzyme to change from the K^+ conformation to a more protected conformation.

Due to the presence of ATP the K^+-specific peptides of 56 and 42 kDa are reduced while peptides with apparent molecular masses of 67 and 35 kDa appear (Fig. 8). As the 55 and 42 kDa peptides do not disappear completely at the ATP concentration used (5 mM) we assume that either this ATP concentration is too low or the conformation obtained in the presence of K^+ plus ATP differs essentially from that in the presence of ATP alone.

Fig. 9 shows an effect of Mg^{2+} that is similar to that of ATP: increasing the Mg^{2+} concentration inhibits the tryptic inactivation in the presence of K^+. Eventually the same inactivation curve is obtained as with Mg^{2+} alone.

The electrophoretic patterns of Fig. 10 show that the effect of Mg^{2+} is also reflected in the

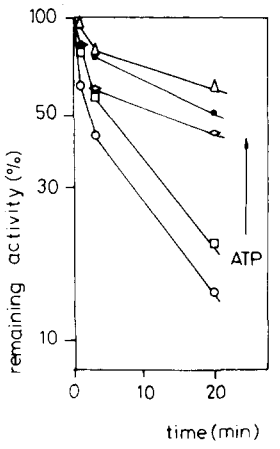


Fig. 7. Effect of ATP on tryptic inactivation of $(K^+ + H^+)$-ATPase activity in the presence of KCl. Incubation was as described in the legend to Fig. 1 with 100 μl containing 150 μg $(K^+ + H^+)$-ATPase, 1.6 μg trypsin, 100 mM KCl alone (○———○) or in combination with ATP in the concentration: 1 mM (□———□), 2 mM (◇———◇) or 5 mM (●———●). The incubation with 5 mM ATP plus 100 mM choline chloride in the medium is given by (△———△).

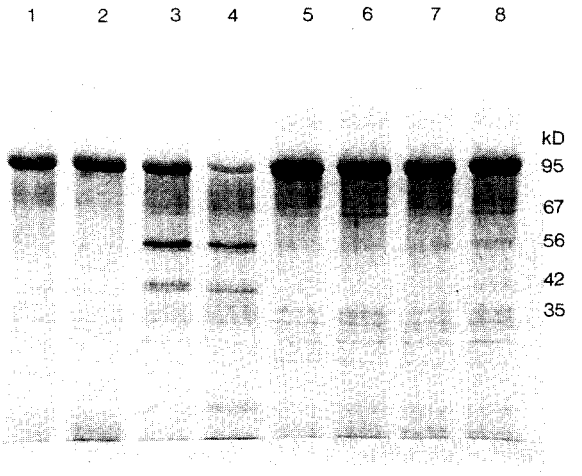


Fig. 8. Electrophoretic pattern of peptides obtained after digestion in the presence of various combinations of ligands as indicated. Trypsin digestion was for 5 min (slots 1, 3, 5, 7) or 20 min (slots 2, 4, 6, 8) in the absence (1, 2) or presence of trypsin (3–8). Incubation occurred in the presence of 100 mM KCl (3, 4); 10 mM ATP, 1 mM EDTA plus 100 mM choline chloride (5, 6); 10 mM ATP plus 100 mM KCl (7, 8). Further conditions were as described in the legend to Fig. 1.

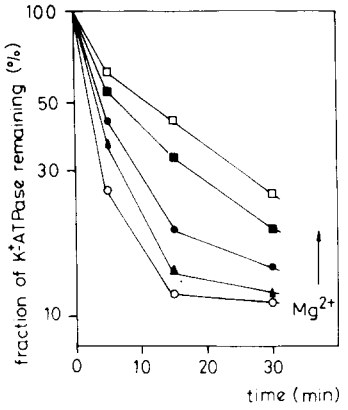


Fig. 9. Effect of Mg^{2+} on tryptic inactivation of $(K^+ + H^+)$-ATPase activity in the presence of KCl. Incubation was as described in the legend to Fig. 7 with 10 mM instead of 100 mM KCl and Mg^{2+} in the concentrations: 0 mM (○———○), 3 mM (▲———▲), 5 mM (●———●) or 10 mM (■———■). The incubation in the presence of 10 mM Mg^{2+} alone is given by (□———□) ionic strength of the incubation media was kept constant by adding choline chloride up to 50 mM.

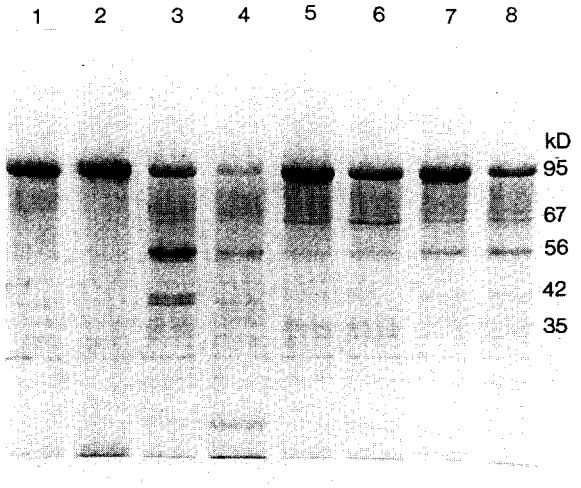


Fig. 10. Electrophoretic pattern of peptides obtained after digestion in the presence of combinations of K^+ and Mg^{2+} ions. Trypsin digestion was for 5 min (slots 1, 3, 5, 7) or 20 min (slots 2, 4, 6, 8) in the absence (1, 2) or presence of trypsin 16 μg/ml (3–8). The $(K^+ + H^+)$-ATPase concentration was 1,5 mg/ml. Incubation occurred in the presence of 10 mM KCl (3, 4); 10 mM $MgCl_2$ plus 70 mM choline chloride (5, 6); 10 mM KCl, 60 mM choline chloride plus 10 mM $MgCl_2$. Further conditions were as described in the legend to Fig. 1.

appearance of a different peptide pattern. The 56 and 42 kDa peptides, specific for the K^+ conformation, are decreased in favour of 67 and 35 kDa peptides. Apparently Mg^{2+} converts the enzyme from a K^+ conformation to a different conformation which is more protected against tryptic inactivation. Considering the appearance of 67 and 35 kDa peptides Mg^{2+} has effects analogous to those of ATP.

Heterogeneity of the main protein chain of $(K^+ + H^+)$-ATPase

We have studied the correlation of the loss of enzyme activity and the disappearance of the 95 kDa chain of $(K^+ + H^+)$-ATPase during tryptic digestion in the presence of K^+ (Fig. 11). The percentage of protein remaining in the 95 kDa band has been determined by scanning. Fig. 11 shows that a linear relationship exists between the residual enzyme activity and protein in the 95 kDa band.

Extrapolation of the curve in Fig. 11 indicates that only 8% of the 95 kDa protein is left when the ATPase activity is reduced to zero. This finding is in disagreement with the observation of Saccomani et al., who found that 34% of the main

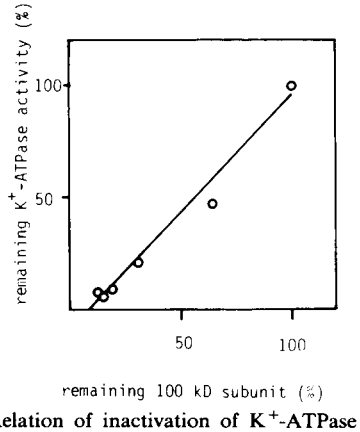


Fig. 11. Relation of inactivation of K^+-ATPase activity and disappearance of 100 kDa subunit of $(K^+ + H^+)$-ATPase. $(K^+ + H^+)$-ATPase was subjected to tryptic digestion in the presence of 100 mM KCl as described in the legend to Fig. 1. At 0, 5, 15, 30, 45 and 60 min of samples were taken from the incubation mixture of which half was used to determine the K^+-ATPase activity (*Y*-axis). The other half of the sample was subjected to polyacrylamide gel electrophoresis in SDS. The amount of protein remaining in the 100 kDa band (*X*-axis) was estimated by scanning as described in Materials and Methods.

peptide remained in the membrane at complete inhibition of K^+-ATPase activity. This was one argument leading them to the conclusion that the main protein chain of $(K^+ + H^+)$-ATPase is heterogeneous.

Another argument for their conclusion stemmed from phosphorylation experiments. After tryptic digestion in the presence of ATP only the main protein chain could be phosphorylated by [γ-^{32}P]ATP while the lower molecular weight peptides could not be labeled. This in contrast with our findings. Fig. 12 shows the electrophoretic pattern of $(K^+ + H^+)$-ATPase preparations, phosphorylated after tryptic digestion. Before phosphorylation the digested preparations were applied to a Sephadex G-25 column, in order to remove the ligands K^+ and ATP. Fig. 12 shows that in the undigested preparation the main peptide chain is phosphorylated and a peptide chain of 150 kDa, which is probably a dimer of the main chain. After tryptic digestion in the presence of K^+, ^{32}P incorporation is observed in the 95 kDa and 42 kDa peptide bands, indicating that the latter fragment still contains the phosphorylation site. After tryptic digestion in the presence of ATP, ^{32}P incorporation is observed in the 95 kDa chain and in a 67 kDa tryptic fragment. The low level of ^{32}P incorporation in this preparation can be accounted for by residual unlabeled ATP in the enzyme preparation.

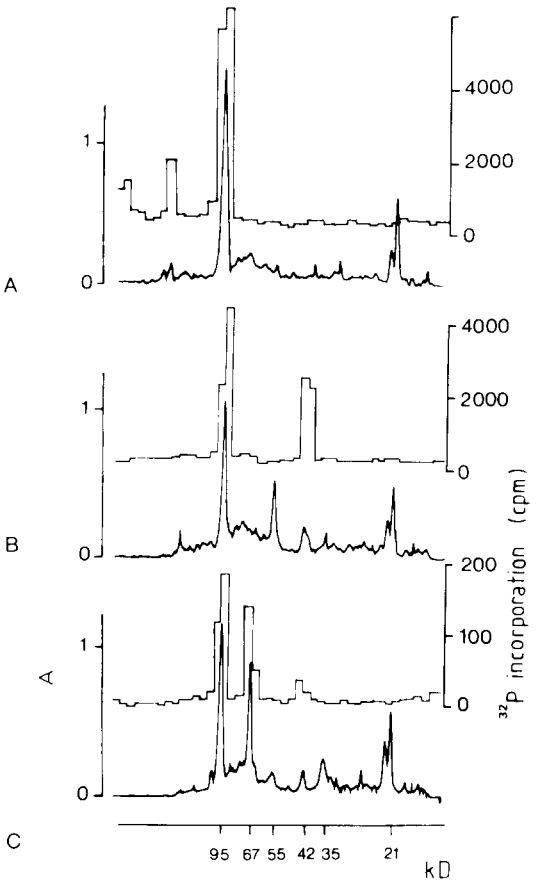


Fig. 12. Phosphorylation capacity of tryptic peptides of $(K^+ + H^+)$-ATPase. $(K^+ + H^+)$-ATPase was subjected to tryptic digestion in the presence of 100 mM K^+ (B) or 5 mM ATP, 1 mM EDTA plus 100 mM choline chloride (C). A control preparation which was not subjected to tryptic digestion is shown in (A). Tryptic digestion occurred during 5 min (B) and 30 min (C) as described in the legend to Fig. 1. Further procedures were as described in Materials and Methods.

These results bring us to the conclusion that all the 95 kDa protein chains contain a phosphorylation site. In combination with the findings of Fig. 10 we conclude therefore that all proteins in the 95 kDa band are catalytic subunits, giving no argument for heterogeneity of the subunits of $(K^+ + H^+)$-ATPase.

Discussion

Tryptic digestion of the 95 kDa chain of the purified membrane bound $(K^+ + H^+)$-ATPase is a slow and specific process. Due to bond splitting at specific sites in the protein chain distinct peptides are formed. The concentration of trypsin has been adjusted so that under the experimental conditions used the trypsin activity, as measured by the digestion of an artificial substrate is always the same. The differences in both inactivation kinetics and the cleavage of the 95 kDa chain are therefore not due to effects of the used ligands on the activity on trypsin. In the presence of K^+ different sites on the protein are exposed than in the presence of ATP, implying that two conformations termed the K^+-conformation and the ATP conformation, can be defined by their characteristic patterns of digestion. The effect of K^+ is not a general ionic effect as in the presence of choline or Na^+ not the same characteristic peptide pattern is obtained.

In the presence of Mg^{2+} a not very distinct peptide pattern is obtained, which is apparently a

combination of the K^+ and the ATP peptide pattern. However, it is shown in two ways that Mg^{2+} has an effect on the enzyme that is similar to that of ATP. The enzyme in a K^+-conformation can be converted to a different conformation by ATP, resulting in a decrease of inactivation (Fig. 7) and a change in the resulting peptide pattern (Fig. 8). Mg^{2+} has an analogous effect, both on the time course of inactivation and the resulting peptide pattern. In both cases the resulting peptide pattern is not completely converted to that in the presence of ATP alone. The tryptic digestion method appears not to be adequate to distinguish a specific Mg^{2+}-conformation. It only shows that the Mg^{2+}-conformation is different from both the K^+- and the ATP-form.

The conformational change induced by ATP requires relatively high concentrations of ATP, suggesting that the K^+-conformation has a low affinity for ATP. ADP has the same effect both on the time course of inactivation and the resulting peptide pattern (not shown) and Mg^{2+} is not necessary for the effect of both ATP and ADP to occur. In fact even in the presence of EDTA, ATP can exert its protective effect.

Another effect of Mg^{2+} is that it supports the protection by ATP against tryptic inactivation. Beside the protective effect of Mg^{2+} has of its own, it also decreases the $K_{0.5}$ value for the protective effect of ATP form 1.6 mM (no Mg^{2+}) to 0.2 mM in the presence of 10 mM Mg^{2+}. As both in the presence of ATP plus Mg^{2+} and with ATP alone phosphorylation occurs [22], it could be proposed that the so-called ATP-conformation reflects the phosphorylated state (EP) of the enzyme. However, with EDTA in addition to ATP in the medium no phosphorylation occurs [24], and as ADP gives the same effect, it seems more likely that a conformation with ATP merely bound (E · ATP) is observed. As binding of ATP necessarily precedes phosphorylation it seems likely that the observed effect of ATP reflects the binding of ATP to the enzyme. Apparently mere binding and phosphorylation cannot be distinguished with this method, unlike to what is the case for ($Na^+ + K^+$)-ATPase [17].

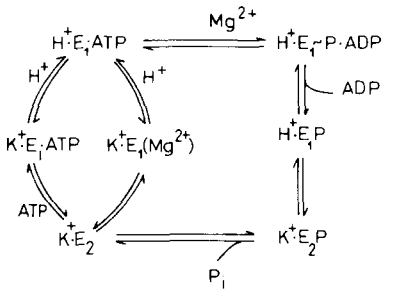
The results obtained correlate well with the present knowledge on the conformational states of the dephosphoenzyme (E_1 and E_2). The E_1 conformation of the enzyme is generally defined as a form with high affinity for ATP and with a low affinity for K^+. The enzyme in an E_1 conformation is rapidly phosphorylated by ATP in the presence of Mg^{2+}. Conversely the E_2 conformation has a low affinity for ATP and high affinity for K^+, which binds to luminal side of the enzyme. These definitions are obtained from experiments on phosphorylation and dephosphorylation of the enzyme, proton transport studies and from experiments with modifying agents [11,14,25–30].

The effects of Mg^{2+}, K^+ and ATP as observed in this paper, fit well in the concept of the enzyme: the enzyme in the K^+-form has a low affinity for ATP; ATP and Mg^{2+} are synergistic in antagonizing the effect of K^+. Whether Mg^{2+} induces an E_1 conformation as defined above cannot be concluded from the experiments in this paper.

The obtained results are in agreement with those of eosin fluorescence in the presence of ($K^+ + H^+$)-ATPase as previously reported by us [13]. In these studies an antagonism between K^+ and Mg^{2+} has been reported and it was shown that Mg^{2+} induces (and K^+ abolishes) an extra binding site for eosin which has been shown to be the high-affinity ATP binding site essential for catalytic activity. The results correlate also with the findings of Schrijen et al. [9] on inactivation of ($K^+ + H^+$)-ATPase by butanedione. In those experiments Mg^{2+} and K^+ have opposite effects in the sense that Mg^{2+} exposes an essential arginine residue, whereas K^+ protects it.

The effect of trypsin on the ($K^+ + H^+$)-ATPase can be accounted for by the given reaction scheme (Scheme I). The sequence is an adaptation of schemes reported by others [25,30]. ATP and Mg^{2+} both induce a conformation which is not an E_2 conformation but also differs from a genuine E_1 conformation in the sense that it has K^+ still bound at sites which are assumed to be low-affinity sites at the luminal side of the membrane. It is assumed that binding of protons, which will occur more readily at low pH, will induce the E_1 conformation completely as has already been suggested by Stewart et al. [28]. After complete inducing of the E_1 conformation rapid phosphorylation by ATP will occur.

A second important observation in this study is

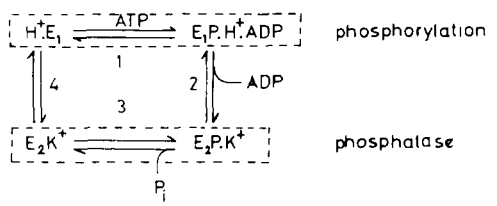


Scheme I.

the way in which the time course of inactivation of the K^+-ATPase, K^+-phosphatase and phosphorylation are correlated. As shown in Fig. 1 and Fig. 2 the time course of inactivation of the K^+-ATPase reaction has a rapid initial phase. During the corresponding period of time the K^+-phosphatase and phosphorylation capacity are not inactivated but even stimulated. Apparently in the first period of the digestion process the K^+-ATPase reaction is interrupted at a step in the cycle which is neither involved in the phosphorylation nor in the phosphatase reaction. Based on the present knowledge of the $(K^+ + H^+)$-ATPase reaction cycle the conversion of the phosphoenzyme (step 2 in the Scheme II) is likely to be that step.

A logic consequence of this would be that after tryptic digestion the obtained phosphoenzyme is more ADP-sensitive and less sensitive to K^+. However, thus far we have not been able to observe a change in the ADP- or K^+-sensitivity. Also the hydroxylamine sensitivity is unchanged.

Comparing the time courses of inactivation with the electrophoretic pattern of the occurring peptides it seems likely that the fast initial inactivation of the K^+-ATPase activity is correlated to the appearance of the large peptides with apparent molecular masses of 72 and 78 kDa. Apparently cleavage of a small fragment from the 95 kDa subunit results in inactivation of the K^+-ATPase activity. Later in the process other cleavage products are formed, which causes the *p*-phenylphosphatase and the phosphorylation reaction to be inactivated too.



Scheme II.

The stimulation of the phosphorylation capacity is in our experiments maximally 10%, whereas Saccomani et al. [5] reported a stimulation of 100% or more. This large stimulation may have been caused by underestimation of the capacity of the control due to suboptimal phosphorylation conditions. Saccomani et al. used a very high protein concentration (0.3 mg/ml), a relatively low ATP concentration (2 μM) and a long incubation time (15 s). When we use a normal, active enzyme for phosphorylation, ATP will under those conditions be exhausted and the measured phosphorylation level will be lower than the optimal steady state phosphorylation level. This will be even more so when K^+ is present during phosphorylation. When the enzyme is inactivated the hydrolysis of ATP will be less and phosphorylation will be the same as the steady state level. Thus the stimulation of 100% or more as observed by Saccomani et al. may be artefactual.

A third important observation made in this study concerns the homogeneity of the main protein chain of the $(K^+ + H^+)$-ATPase preparation. This study does not provide a final solution for the controversion between Saccomani et al. [5] and Peters et al. [6] concerning this problem. The statement of heterogeneity of the subunits by Saccomani et al. was partly based on the observation that 34% of the main protein chain remains undigested at complete inactivation of the K^+-ATPase activity. Moreover, it was found that the peptides obtained after tryptic digestion in the presence of ATP could not be phosphorylated and therefore could not be a catalytic subunit.

Neither of these two observations are confirmed by us (Figs. 11 and 12). Only about 8% of protein remains at complete inhibition of the K^+-ATPase activity (Fig. 12) and a 67 kDa tryptic fragment can be phosphorylated. For this experiment we used a high concentration of [γ-^{32}P]ATP, while removing of excess of unlabeled ATP was necessary. Neither of these conditions were applied by Saccomani et al. which could be the

reason why no labeling of a tryptic fragment could be obtained by them.

Although in the light of our findings we still favour homogeneity of the protein moiety of the main protein subunit of $(K^+ + H^+)$-ATPase, heterogeneity of the sugar residues on the protein is not excluded. This would also explain the heterogeneity in p*I* values of the subunits and their different immunogenicity [7].

If the 95 kDa band is homogeneous with regard to protein, then it is still hard to comprehend why the level of acid-stable phosphoenzyme formed with ATP indicates that only one out of three or four subunits is labeled, as calculated from the maximal steady-state phosphorylation level of 1.5 nmol per mg protein. Further study is needed to solve this problem.

The obtained results may be helpful in elucidating the topography of the main protein chain of $(K^+ + H^+)$-ATPase. Some of the fragments formed after tryptic digestion can still be phosphorylated: a 42 kDa fragment with K^+ present and a 67 kDa fragment with ATP present, indicating that both fragments contain the phosphorylation site.

Recently the amino acid sequence and molecular mass (114012 Da) of the catalytic subunit of the rat gastric $(K^+ + H^+)$-ATPase has been established [8]. From the knowledge on the gene and protein structure of the cation pumps the molecular mass of pig stomach $(K^+ + H^+)$-ATPase is bound to be almost the same. This means that the molecular of the catalytic subunit and probably also of the cleavage products in this study have been underestimated. As discussed before by Peters et al. [6] the high electrophoretic mobility may be due to the relatively high content of hydrophobic amino acids, which can lower the apparent molecular mass obtained from gel electrophoresis [31].

Assuming the main protein chain and the smaller fragments are affected in their electrophoretic mobility to the same degree one can calculate (using a correction factor 114/95) that tryptic digestion in the presence of K^+ yields products of 49 and 65 kDa. In $(Na^+ + K^+)$-ATPase tryptic digestion in the presence of K^+ is between Arg-438 and Ala-439 [32], which site corresponds to Arg-454 and Ile-455 in gastric $(K^+ + H^+)$-ATPase [8]. Splitting in this place would yield cleavage products with molecular masses of 50 and 64 kDa, which is in good agreement with our 'corrected' values. Moreover the phosphorylation site would be in the smaller fragment as we have found.

A similar reasoning for the cleavage products in the presence of ATP yields values for their molecular mass of 39 and 76 kDa. In $(Na^+ + K^+)$-ATPase cleavage of the E_1 form occurs between Arg-278 and Ile-279 [32]. If splitting would occur in $(K^+ + H^+)$-ATPase on the corresponding site the phosphorylation site would be on the larger fragment as we have indeed found, but the molecular masses of the fragments would be 30 and 84 kDa which is somewhat different from the values of 39 and 76 kDa given above. A tentative scheme on the structure of the main subunit indicating phosphorylation and tryptic splitting sites, based on the above reasoning, is given in Fig. 13. A more reliable method for molecular mass determination combined with analysis of the amino acid sequence of the cleavage products will give more detail about the location of the tryptic cleavage sites in the various conformational states of the enzyme.

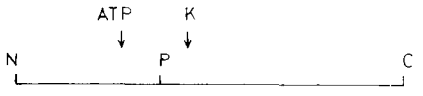


Fig. 13. Tentative scheme of the location of phosphorylation and tryptic cleavage sites on the $(K^+ + H^+)$-ATPase subunit. Through combination of experimental data in this study and knowledge on the primary structure of rat gastric $(K^+ + H^+)$-ATPase [8] and on the tryptic splitting sites on $(Na^+ + K^+)$-ATPase [32] a model for the structure of the $(K^+ + H^+)$-ATPase catalytic subunit has been constructed as described in the text.

Acknowledgements

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