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Structural organization of alpha-subunit from purified and microsomal toad kidney ($Na^+ + K^+$)-ATPase as assessed by controlled trypsinolysis

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The membrane organization of the alpha-subunit of purified ($Na^+ + K^+$)-ATPase (($Na^+ + K^+$)-dependent adenosine triphosphate phosphorylase, EC 3.6.1.3) and of the microsomal enzyme of the kidney of the toad *Bufo marinus* was compared by using controlled trypsinolysis. With both enzyme preparations, digestions performed in the presence of Na^+ yielded a 73 kDa fragment and in the presence of K^+ a 56 kDa, a 40 kDa and small amounts of a 83 kDa fragment from the 96 kDa alpha-subunit. In contrast to mammalian preparations (Jørgensen, P.L. (1975) Biochim. Biophys. Acta 401, 399–415), trypsinolysis of the purified amphibian enzyme led to a biphasic loss of ($Na^+ + K^+$)-ATPase activity in the presence of both Na^+ and K^+. These data could be correlated with an early rapid cleavage of 3 kDa from the alpha-subunit in both ionic conditions and a slower degradation of the remaining 93 kDa polypeptide. On the other hand, in the microsomal enzyme, a 3 kDa shift of the alpha-subunit could only be produced in the presence of Na^+. Our data indicate that (1) purification of the amphibian enzyme with detergent does not influence the overall topology of the alpha-subunit but produces a distinct structural alteration of its N-terminus and (2) the amphibian kidney enzyme responds to cations with similar conformational transitions as the mammalian kidney enzyme. In addition, anti alpha-serum used on digested enzyme samples revealed on immunoblots that the 40 kDa fragment was better recognized than the 56 kDa fragment. It is concluded that the NH_2-terminal of the alpha-subunit contains more antigenic sites than the COOH-terminal domain in agreement with the results of Farley et al. (Farley, R.A., Ochoa, G.T and Kudrow, A. (1986) Am. J. Physiol. 250, C896–C906).

Introduction

The plasma membrane enzyme, ($Na^+ + K^+$)-ATPase, is the transport system which regulates the intracellular and extracellular Na^+ and K^+ equilibrium. In its purified form, the enzyme consists of two transmembraneous subunits, an alpha-subunit with a molecular mass of about 112 kDa, which catalyzes the main functional activities, and a glycosylated beta-subunit with a molecular mass of 35–60 kDa (for review, see Refs. 1 and 2). Although it is established that the minimal functional enzyme unit consists of an alpha-beta dimer [3], the role played by the beta-subunit in the transport activities remains unknown.

($Na^+ + K^+$)-ATPase can be purified in a membrane-bound, active form [4] and these char-

Abbreviations ($Na^+ + K^+$)-ATPase, ($Na^+ + K^+$)-dependent triphosphate phosphorylase (EC 3.6.1.3), SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Mg^{2+}-ATPase, Mg^{2+}-dependent adenosine triphosphate phosphorylase, PMSF, phenylmethylsulfonyl fluoride

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acteristics render these preparations ideal for the study of the enzyme's structural and functional properties. Indeed, studies using controlled proteolysis in combination with chemical labeling of the purified enzyme have led to a model of the membrane arrangement of the alpha-subunit [5], which in general is consistent with the transmembrane topology proposed on the basis of the amino acid sequence of the polypeptide [6,7]. In addition, reconstitution of the purified enzyme in lipid vesicles have brought valuable data on the transport properties of the $(Na^+ + K^+)$-ATPase (for review, see Refs. 8 and 9). One potential drawback of these experimental approaches is, however, that purification of $(Na^+ + K^+)$-ATPase can only be achieved by using significant concentrations of sodium dodecyl sulfate (SDS) [4]. It is indeed conceivable that the detergent might alter the native membrane organization of the enzyme and in consequence modify its physiologically relevant operational state. Thus, for a better definition of the complex functions of this enzyme, it is important to study the membrane arrangement of the purified enzyme and to compare it to the enzyme configuration in preparations free of detergents.

In the present study we have compared a SDS-purified enzyme preparation with a detergent-free microsomal fraction of toad kidney and have used controlled trypsinolysis as an experimental tool to gain information on the membrane organization of the polypeptides as well as on their functional state Indeed, it has been shown that the characteristic proteolytic fragmentation of the alpha-subunit in response to Na^+ or K^+ reflects the cation-induced conformational transitions of the active enzyme [10,11]. The specificity of controlled trypsinolysis of $(Na^+ + K^+)$-ATPase has been assessed in two ways: (i) by comparing the time-course of $(Na^+ + K^+)$-dependent ATPase inactivation to the degradation of the catalytic alpha-subunit of the enzyme; (ii) by identifying the specific cation-dependent tryptic fragments, using protein staining on SDS gels or immunoblotting revealed with monospecific polyclonal antibodies raised against the alpha-subunit of $(Na^+ + K^+)$-ATPase [12]

The data of this study indicate that structural features of the amphibian microsomal enzyme compare well to purified mammalian preparations but that SDS treatment of crude amphibian microsomes abolishes cation modulation of the N-terminus of the amphibian $(Na^+ + K^+)$-ATPase.

Experimental Procedures

Microsomal fractions and purified $(Na^+ + K^+)$-ATPase from the kidney of the toad *Bufo marinus* (obtained from C.P. Chase, Miami, FL) were prepared as described [12] by using a modified procedure originally developed by Jørgensen [4]. Protein content was determined by the method of Lowry et al. [13] and enzyme activity according to previously described methods [14,15]. Microsomal and purified enzyme preparations had specific activities of 20 to 30 and 600 to 900 μmol $P_i \cdot (mg\ protein)^{-1} \cdot h^{-1}$, respectively. Mg^{2+}-ATPase measured in the presence of 10^{-4} M ouabain made up about 80% and 3% of the total ATPase activity in microsomal fractions and purified enzyme preparations, respectively.

Controlled proteolysis of $(Na^+ + K^+)$-ATPase

Purified or microsomal $(Na^+ + K^+)$-ATPase was digested on ice in an incubation medium A containing 150 mM KCl or 150 mM NaCl, 18 mM Tris-HCl (pH 7.4), 30 mM DL-histidine and 5 mM EDTA, either in the presence of trypsin (Sigma type XI) or proteinase K (Merck). Incubations with trypsin proceeded at 0 °C or 25 °C at protease to protein ratios (w/w) between 0,2 to 10 in the presence of Na^+ or K^+ for various time periods indicated in the figures. Proteinase K was used at a protease to protein ratio of 2.5 Control samples were incubated in parallel in the absence of proteases. Trypsin digestion was stopped by addition of a 5-fold excess of soybean trypsin inhibitor (Sigma) and proteinase K digestion by 2 mM phenylmethylsulfonyl fluoride (Merck, Stock solution: 200 mM in 90% ethanol). By adding proteinase inhibitors to the samples before addition of proteases, it could be shown that digestion stop occurred immediately. Routinely, samples were left on ice for 10 min after addition of the inhibitor. The control and digested preparations were then centrifuged in thick wall polycarbonate tubes (Beckman) in a SW55 rotor in a Beckman LB-70 ultracentrifuge at $100\,000 \times g$ for

60 min at 4°C. The pellets were resuspended in incubation medium A and aliquots were taken for activity measurements. The remaining preparation was recentrifuged and the final pellet taken up in sample buffer (4.5% SDS, 8.3% sucrose, 0.012% bromophenol, 2% beta-mercaptoethanol), boiled for 5 min and 6–48 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE).

Gel electrophoresis and Western blot

SDS-PAGE was performed as described [14]. Purified $(Na^+ + K^+)$-ATPase was detected by Coomassie blue (R250, Merck) and quantitated by laser densitometry (LKB 2202, Ultrascan). It was established that peak heights of alpha-subunit of $(Na^+ + K^+)$-ATPase were linearly related to the amount of purified enzyme layered on the gel (up to 50 μg of protein) when gels were scanned before drying.

Alternatively, samples of microsomal fractions or of the purified enzyme run on 5–13% SDS polyacrylamide gels were transferred to nitrocellulose filters [16,17] and stained with Coomassie blue [18] or Ponceau red [19]. After 1 h of incubation in phosphate-buffered saline (PBS) containing 5% newborn calf serum, the blots were incubated overnight with anti alpha-subunit serum diluted 1 : 100 in phosphate-buffered saline containing 0.5% Tween-20. After several washes, the bound antibodies were overlayed with protein A-peroxidase conjugate (Sigma) and revealed with 4-chloronaphthol and H_2O_2. The revealed bands were quantitated by laser densitometry.

Anti alpha-subunit serum

The anti alpha-subunit serum was prepared as previously described [12] by using as antigen gel pieces containing the alpha-subunit resolved on SDS-PAGE from purified enzyme preparations. For overlay of Western blots of $(Na^+ + K^+)$-ATPase, the antiserum was preincubated for 2 h at 37°C and 2 h at 4°C with 10% toad serum, centrifuged at 110000 × *g* for 30 min at 4°C and filtered on a 0.22 μm Millipore filter. The antiserum was stored at −20°C.

Results

(1) Tryptic digestion of alpha-subunit of $(Na^+ + K^+)$-ATPase purified from the kidney of Bufo marinus

Since most of the previous studies which can

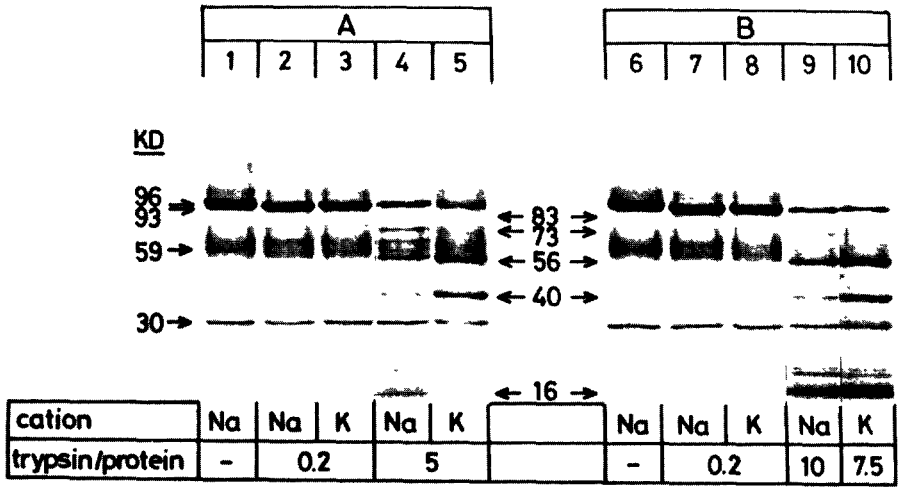


Fig 1 Trypsinolysis of purified toad kidney $(Na^+ + K^+)$-ATPase Purified $(Na^+ + K^+)$-ATPase (48 μg protein) was incubated with indicated concentrations of trypsin in the presence of 150 mM NaCl or KCl for (A) 20 min at 25°C or (B) 60 min at 0°C The samples were prepared for SDS-PAGE and the gels stained with Coomassie blue as described in Experimental Procedures. 96 KD = 96 kDa, for example Molecular masses of the proteolytic fragments were determined by using six protein standards with known molecular weights (Sigma).

serve as a reference had been performed on the purified enzyme of mammalian sources we first characterized the purified toad kidney ($Na^+ + K^+$)-ATPase in order to compare the amphibian to the mammalian enzyme.

As shown in Fig. 1A (lane 1), purified ($Na^+ + K^+$)-ATPase of the toad kidney with a mean specific activity of 700 μmol $P_i \cdot (mg\ protein)^{-1} \cdot h^{-1}$ is mainly composed of the 96 kDa alpha-subunit and the 60 kDa glycoproteinic beta-subunit. The identity of these two subunits of ($Na^+ + K^+$)-ATPase has prevously been determined [14]. Due to the poor and diffused staining of the heavily glycosylated beta-subunit by Coomassie blue, it is, however, impossible to give a reliable estimate of the degree of purity of this enzyme preparation. Besides the two ($Na^+ + K^+$)-ATPase subunits, a 30 kDa polypeptide was consistently co-purified and it is not yet established whether this protein is part of the active enzyme unit or a contaminant.

In addition to low molecular weight contaminants, the gel pattern of the purified enzyme frequently revealed small amounts of two polypeptides, the molecular masses of which namely 59 kDa and 41 kDa, approximately summed up to the molecular mass of the alpha-subunit. We propose that these latter polypeptides represent spontaneously produced proteolytic fragments of the catalytic subunit (for further evidence, see below).

The purified enzyme preparation was subjected to controlled trypsinolysis to reveal structural properties of the alpha-subunit which could serve as a basis for comparison with other purified and characterized ($Na^+ + K^+$)-ATPase preparations on the one hand and with the non-detergent-treated enzyme from microsomal kidney fractions or homogenates of cultured cells on the other hand. The procedure is known to yield characteristic proteolytic frgments, the appearance of which depends on the difference conformations adopted by the membrane-bound active enzyme in the presence of either Na^+ or K^+ [10].

The alpha-subunit of purified toad kidney ($Na^+ + K^+$)-ATPase showed to be quite resistant to the action of trypsin. No significant amounts of Na^+ or K^+ specific proteolytic fragments of the alpha-subunit could be produced when the enzyme was incubated for 20 min at 25°C with trypsin to protein ratios of 0.2 (Fig. 1, lanes 2 and 3). Such treatment only resulted in a shift of the total population of 96 kDa subunit into a 93 kDa polypeptide and the parallel disappearance of the spontaneous degradation fragment of 59 kDa, irrespective of the ionic condition. Significant digestion of the alpha-subunit and thus revelation of the cation-induced conformational states could, however, be obtained with trypsin to protein ratios of 1 to 5 (Fig. 1, lanes 4 and 5). These conditions led to the production of a 73 kDa fragment which was more prominent in the presence of Na^+ (Fig 1, lane 4) than in the presence of K^+ (Fig. 1, lane 5). On the other hand, the formation of a 56 kDa and a 40 kDa fragment was more important in the K^+ than in the Na^+ condition. In addition, some secondary cleavage products of low molecular mass appeared in both ionic conditions. Neither the beta-subunit, nor the 30 kDa polypeptide were digested under these conditions.

Since one reason for the characterization of the purified toad kidney enzyme was to create a reference for comparison with the enzyme from cell homogenates, we performed further experiments at 0°C rather than at 25°C Indeed, we observed, that experiments done on cell homogenates required working at 0°C in order to avoid the activation of cell specific proteases [20].

Incubation of the purified enzyme for 60 min at 0°C at a trypsin to protein ratio of 0.2 in fact produced a very similar tryptic pattern as at 25°C (Fig. 1, compare lanes 2 and 3 with 7 and 8). In addition, despite a probable impairment of ion-specific conformational changes at 0°C, it was possible, after increase of the trypsin to protein ratio, to reveal the adoption of different configurations in response to Na^+ and K^+ through the production of the cation-specific proteolytic fragments. In particular, a more prominent production of the 56 kDa and 40 kDa fragment could be observed in the K^+ condition than in the Na^+ condition (Fig. 1, lanes 9 and 10).

The time-courses of trypsinolysis at 0°C at trypsin to protein ratios of 10 and 7 5 in the presence of Na^+ and K^+, respectively, revealed that. (1) the 3 kDa shift of the alpha-subunit was produced within a few minutes in both conditions, (2) the enzyme in the Na^+ conformation was more resistant to trypsinolysis, leaving about 30% of the

alpha-subunit in the 93 kDa form after 60 min of trypsinolysis compared to about 15% in the K^+ conformation, (3) the 73 kDa fragment (characterized as Na^+ specific in trypsinization experiments performed at 25 °C, see Fig. 1A, lane 4) was produced more rapidly and to a greater extent in the Na^+ condition than in the K^+ condition, (4) during a 60 min trypsinolysis, secondary tryptic fragments were formed which differed in the two ionic conditions (Fig. 2A + B).

In addition to the production of the described tryptic fragments of 93, 73, 56 and 40 kDa, the molecular masses of which closely resemble the ones described for mammalian enzymes, and some secondary cleavage products of low molecular mass, the toad kidney preparation yielded small amounts of an 83 kDa fragment exclusively in the K^+ condition (Fig. 1, lane 10 and Fig. 2). Proteinase K, which is less specific than trypsin, produced at protease to protein ratios of 2.5, significant quantities of this product (see below and Fig. 5) and thus it represents most likely a specific cleavage product of the alpha-subunit in the K^+ configuration and not of a minor contaminant comigrating with the alpha-subunit.

(2) Correlation between trypsinolysis of the alpha-subunit and the loss of $(Na^+ + K^+)$-ATPase activity

Further indications on the homogeneity of the

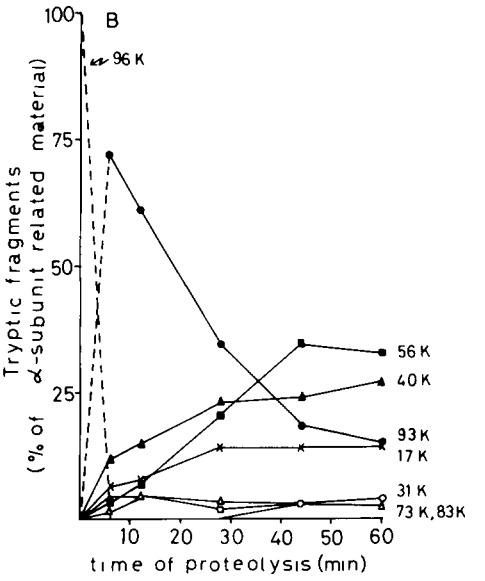
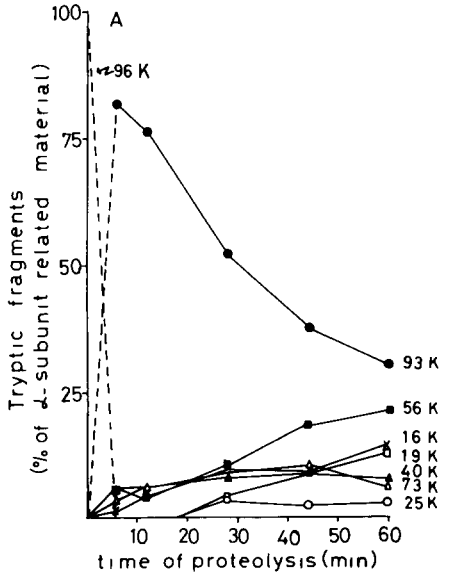


Fig 2 Time-course of tryptic digestion of alpha-subunit of purified $(Na^+ + K^+)$-ATPase from toad kidney $(Na^+ + K^+)$-ATPase was exposed for indicated times to trypsin at 0 °C, prepared for SDS-PAGE and the proteolytic fragments quantitated on Coomassie blue stained gels as described in Experimental Procedures The graphs show the relative proportions of individual tryptic fragments in the total alpha-subunit related material at various times after trypsinolysis The dotted lines represent the hypothetical time-course of the shift of the 96 kDa alpha-subunit into the 93 kDa cleaved form (A) Trypsinization in the presence of 150 mM NaCl at a trypsin to protein ratio of 10. (B) Trypsinization in the presence of 150 mM KCl at a trypsin to protein ratio of 7 5



alpha-subunit were obtained by following the relation existing between the decrease of the alpha-subunit population and the loss of enzyme activity during trypsinolysis. The expectation was that the loss of enzyme activity would closely parallel the disappearance of the alpha-subunit during trypsinolysis. As can be seen in Fig. 3 A + B, the semi-logarithmic curve, describing the decrease in the enzyme activity during trypsinolysis in the presence of Na^+ and K^+ was biphasic. Within a few minutes of trypsinolysis, the enzyme activity decreased to about 60% of the control value. From then on, it diminished more slowly and as shown for the K^+ condition was abolished after about 3 h of digestion (Fig. 3B). In the initial rapid phase, the loss of enzyme activity coincided with the complete cleavage of the 96 kDa alpha-subunit population to the 93 kDa form in both ionic

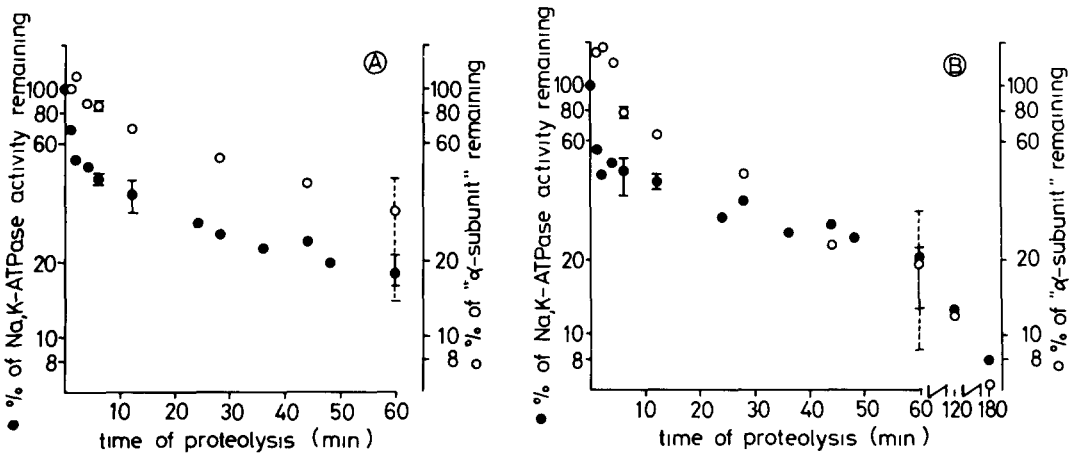


Fig 3 Relation between the disappearance of alpha-subunit and loss of $(Na^+ + K^+)$-ATPase activity during trypsinolysis in the presence of Na^+ and K^+ Digestion of alpha-subunit was followed in the presence of 150 mM NaCl (A) or 150 mM KCl (B) as described in Fig 2 and in Experimental Procedures 'Alpha-subunit' (○). 96 kDa or 93 kDa cleaved form (for further explanation see text) Represented are the percentages of the 96 kDa and 93 kDa forms, respectively, of the alpha-subunit at various time points of trypsinolysis compared to a control sample incubated for 60 min in the absence of trypsin At each time-point of trypsinolysis, $(Na^+ + K^+)$-ATPase activity (●) was determined in the same samples as described in Experimental Procedures and expressed as percentage of the activity of a control sample incubated for 60 min in the absence of trypsin At time points of trypsinolysis where several experiments were performed, the mean value and the range of the mount of the 'alpha-subunit' (- - - - -) and of the remaining enzyme activity (————) are indicated. At 6 min of digestion $n = 4$, at 60 min of digestion, $n = 8$

conditions (Fig. 2 A + B) with only a minor decrease in the absolute intensity of the Coomassie blue stained polypeptide (Fig. 3 A + B). (Since between 0 and 4 min of trypsinolysis, both 96 kDa and 93 kDa polypeptides were produced which could, however, not be separately quantified, we refer to the 96 and 93 kDa region of the gel as the 'alpha-subunit' region in Fig. 3).

Once the 3 kDa shift was complete, e.g. after 6 min of trypsinolysis, the amount of the 93 kDa form of the alpha-subunit diminished in parallel to the enzyme activity of the preparation (Fig. 3). In addition, as shown in Fig. 3B, complete digestion of the 93 kDa polypeptide coincided with the complete loss of enzyme activity.

A similar biphasic enzyme inactivation was observed with mammalian preparations but only in the Na^+ conformation of the enzyme [10,11]. It was concluded that the initial cleavage process which removes a 2 kDa fragment from the N-terminal of the alpha-subunit [21,23] gives rise to a polypeptide with impaired enzymatic activity [10].

Thus, our results indicate that the actual activity of the enzyme preparation is intimately related to the amount of 96 kDa or 93 kDa material, respectively.

(3) Comparison of structural features of SDS-purified and microsomal $(Na^+ + K^+)$-ATPase by polyclonal antibodies

In order to test whether detergent-treatment might modify the native membrane topology of $(Na^+ + K^+)$-ATPase and thus its inherent functional properties, we compared the tryptic fragmentation of the alpha-subunit derived from the SDS-purified enzyme with the one obtained from microsomal fractions devoid of detergent.

Since tryptic products of the alpha-subunit obtained from microsomal fractions could not be reliably detected on Coomassie blue stained gels, we used a polyclonal anti alpha-subunit serum for their revelation after gel migration and blotting of the digested preparations to nitrocellulose paper

As can be seen in Fig. 4, immunoblotting with this antiserum of the SDS-purified enzyme indeed revealed the major proteolytic fragments characterized above on Coomassie blue stained gels. Thus, the 96 kDa alpha-subunit and the spontaneously produced 59 kDa fragments were detected

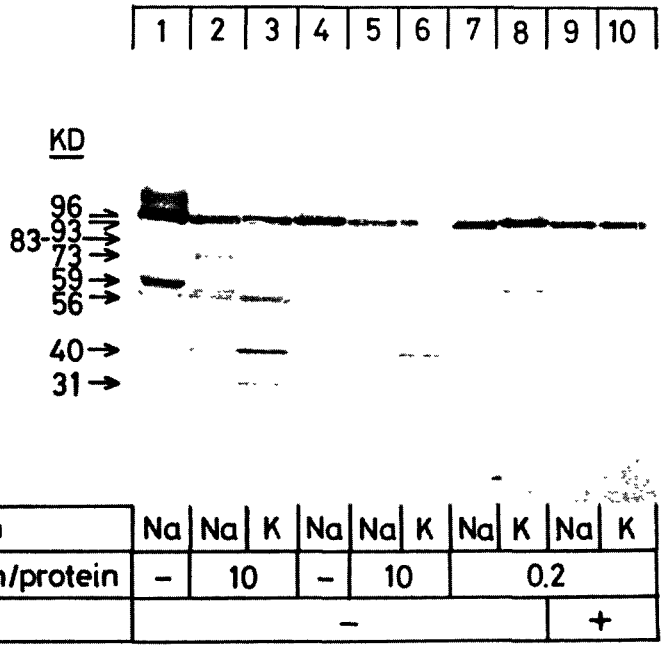


Fig 4 Comparison of the tryptic fragmentation of alpha-subunit from purified and microsomal ($Na^+ + K^+$)-ATPase Purified ($Na^+ + K^+$)-ATPase (5 μg protein) (lanes 1–3) or microsomal kidney fractions (15 μg protein) (lanes 4–10) were treated with trypsin at indicated concentrations in the presence of 150 mM NaCl or KCl for 60 min at 0 °C Samples were then run on SDS-PAGE, transferred to nitrocellulose filters and revealed with anti-alpha-subunit serum as described in Experimental Procedures DOC deoxycholate, used at a detergent to protein ratio of 0 2

in non-digested samples (Fig. 4, lane 1) as well as the Na^+-specific 73 kDa (Fig. 4, lane 2) and the K^+ specific 83, 56 and 40 kDa fragments (Fig. 4, lane 3) in samples treated with trypsin at a trypsin to protein ratio of 10. In addition, in the K^+ conditioned samples, one of the secondary proteolytic fragments of 31 kDa was recognized by the antiserum. Nothing can be said on the immunoreactivity of the lower molecular mass fragments produced in the various digestion conditions (Fig. 2 A + B), since they were very poorly transferred from the 10–13% polyacrylamide gel region to the nitrocellulose support.

When microsomal fractions were treated with trypsin under identical conditions, the proteolytic fragmentation of the alpha-subunit was very similar to the one produced from the purified enzyme (Fig. 4, lanes 4–6). Indeed, in both the Na^+ and K^+ conditions, the 96 kDa alpha-subunit was shifted by 3 kDa and the same Na^+- and K^+-specific fragments were revealed as with the purified enzyme. Only when trypsin to protein ratios were lowered to 0.2, a slight difference in trypsin sensitivity of the two preparations became visible. Thus, while with the purified enzyme, the 3 kDa shift of the alpha-subunit occurred in both the Na^+ and K^+ configurations under these digestion conditions (see above and Fig. 1, lanes 6 versus 7 and 8), with the detergent-free microsomal enzyme, this cleavage took place only in the Na^+ condition (Fig. 4, lanes 7 and 8) Since inclusion during proteolysis of small amounts of detergent (e.g. deoxycholate at a detergent to protein ratio of 0.2) rendered the preparations sensitive to the 3 kDa cleavage in the two ionic conditions (Fig. 4, lanes 9 and 10), it can be concluded that the segment of the alpha-subunit containing this tryptic site is indeed readily affected by detergent treatment.

In mammalian preparations of purified ($Na^+ + K^+$)-ATPase, a similar 2 kDa cleavage site has been described [23] and by sequence analysis has been assigned to the N-terminal of the alpha-subunit [21]. In view of these data, the result obtained

under mild digestion conditions (trypsin to protein ratio 0.2) of the microsomal enzyme also suggest that the 59 kDa spontaneous fragment visible with non-trypsinized samples derives from the N-terminal of the alpha-subunit e.g. of the same segment which contains the 3 kDa cleavage site. Indeed when the 96 kDa alpha-subunit was cleaved into the 93 kDa polypeptide, namely in the Na^+ condition in the absence or presence of detergent (Fig. 4, lanes 7 and 9) or in the K^+ condition in the presence of detergent (Fig. 4, lane 10) the 59 kDa fragment was cleaved in parallel. On the other hand, no digestion of this fragment occurred in the K^+ condition without detergent in which the alpha-subunit resisted to the 3 kDa cleavage (Fig. 4, lane 8).

(4) Immunoreactivity of the N-terminal versus the C-terminal of the alpha-subunit

Controlled trypsinolysis permitted us to test the antigenicity of various segments of the alpha-subunit. For this purpose, we compared the intensity of the immunoblotted fragments of the alpha-subunit from purified preparations with parallely run nitrocellulose strips containing identical samples but stained with Coomassie blue. As shown in Fig 5, the largest tryptic fragments namely the 83 kDa fragment produced with trypsin or proteinase K^+ (Fig. 5, lane 3) and the 73 kDa fragment produced with trypsin in the presence of NaCl (Fig. 5, lane 2) are as expected best recognized compared to the actual amount of proteins present (compare Fig. 5, lanes 2 A + B, 3 A + B). On the other hand, while trypsinolysis in the presence of KCl produces about the same amounts of the complementary 40 and 56 kDa fragments as revealed by Coomassie blue staining (Fig. 5, lane 1 A), the 56 kDa fragment is less recognized by the antiserum than the 40 kDa fragment (Fig. 5, lane 1B)

Since it has been shown by others that the 40 kDa fragment includes the N-terminal domain [21], it might be concluded that this region contains more antigenic sites than the C-terminal domain of the alpha-subunit.

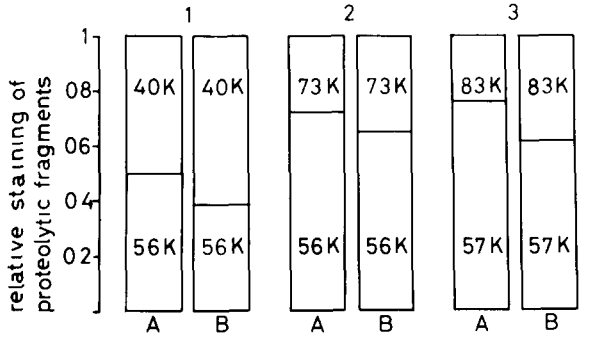


Fig. 5 Relative immunoreactivity of proteolytic fragments of the alpha-subunit Purified $(Na^+ + K^+)$-ATPase was treated with trypsin at a trypsin to protein ratio of 7 5 in the presence of KCl (lane 1), at a trypsin to protein ratio of 10 in the presence of NaCl (lane 2) or with proteinase K at a protease to protein ratio of 2 5 Incubation proceeded at 0 °C for 60 min in the case of trypsin or for 20 min in the case of proteinase K 8 μg of digested samples were divided into two equal aliquots and subjected in parallel to SDS-PAGE and Western blotting. Half of the samples were stained with Coomassie blue (A), and the other half with 4-chloronaphthol/H_2O_2 after overlay of the nitrocellulose with anti alpha-subunit serum (B) Shown are the relative staining intensities of the major proteolytic fragments produced in the different digestion conditions as assessed by laser densitometry 40 K = 40 kDa fragment

Discussion

In the present study, we have characterized the purified $(Na^+ + K^+)$-ATPase from an amphibian source in order to establish a basis for comparison of some structural and functional features of the enzyme with corresponding mammalian preparations on the one hand and with detergent-free enzyme preparations on the other hand.

(1) Purified amphibian $(Na^+ + K^+)$-ATPase displays similar structural features as the mammalian enzyme

Analysis of the structure-function relationship of $(Na^+ + K^+)$-ATPase has mainly been performed on purified mammalian enzyme preparations. In the present paper we extended such studies to the enzyme from an amphibian source and we could show that mammalian and amphibian $(Na^+ + K^+)$-ATPase compare rather well e.g. with respect to their ability to perform cation-induced conformational changes as defined for mammalian preparations by Jørgensen [10].

Indeed, tryptic cleavage products obtained from the alpha-subunit of the amphibian enzyme namely the 73 kDa fragment in the E_1Na form and the 56 and 40 kDa fragments in the E_2K form (Fig. 6)

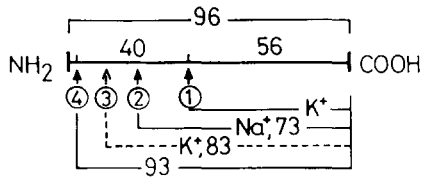


Fig 6 Linear model of alpha-subunit from toad kidney ($Na^+ + K^+$)-ATPase with positioned tryptic sites exposed in the E_1Na or the E_2K form of the enzyme Tryptic fragments are indicated by their molecular mass, (93 = 93 kDa for instance) 1, 2, 3, 4, = tryptic bonds. Bond 1 is predominantly cleaved by trypsin in the presence of KCl in the microsomal and purified enzyme Bond 2 is a minor tryptic site exposed in the presence of KCl Bond 3 is cleaved in the presence of NaCl in the microsomal and the purified enzyme Bond 4 is rapidly cleaved at low trypsin concentrations in the presence of NaCl and KCl in the detergent-treated enzyme but only in the presence of NaCl in the microsomal enzyme

correspond in all likelihood to the similar molecular mass fragments observed with different mammalian preparations [10,21,22]. Our results thus suggest that the accessibility of trypsin to specific amino acids in the polypeptide and in consequence the structural organization of the alpha-subunit of ($Na^+ + K^+$)-ATPase is highly conserved throughout evolution.

Besides the described similarities, the toad kidney ($Na^+ + K^+$)-ATPase might bear, however, some intrinsic structural differences compared to the mammalian kidney preparations. Indeed, the amphibian enzyme exposes an additional tryptic site which has not been described in mammalian preparations and which gives rise although in small amounts to an 83 kDa tryptic product (Fig. 6). The fragment is likely to be a specific cleavage product of the alpha-subunit since (1) its production is dependent on the presence of K^+, a fact which excludes e.g. an identity to a similar tryptic fragment of 90 kDa produced by Ca^{2+}-ATPase samples [24], (2) in proteinase K-treated samples, the 83 kDa fragment makes up an important proportion of the tryptic pattern, a fact which can only be reconciled with cleavage of the alpha-subunit, (3) our data on enzyme inactivation during trypsinolysis clearly show that enzyme activity is inherent to all protein material in the alpha-subunit gel region, (4) the 83 kDa fragment can be revealed as a major tryptic product of the mature enzyme in cell homogenates and is also preferentially produced in K^+ conditions [20].

Only when the amino acid sequence of the amphibian alpha-subunit is determined and can be compared to the one recently established from the cDNA sequence from sheep kidney [7] and from *Torpedo californica* [6] will it be possible to decide whether the exposure of an additional tryptic bond observed in the amphibian enzyme is due to a sequence difference or rather to a difference in the structural organization of the alpha-subunit in different species.

(2) The structure-function relationship of purified ($Na^+ + K^+$)-ATPase compared to detergent-free enzyme preparations

The structure-function relationship of ($Na^+ + K^+$)-ATPase has mainly been studied with purified and thus detergent-treated enzyme preparations. A purpose of the present study was to determine whether data obtained with such preparations are comparable to those obtained with the microsomal enzyme e.g. whether detergent-treatment influences or not the membrane topology and thus the functional properties of ($Na^+ + K^+$)-ATPase. The membrane arrangement of the detergent-free microsomal kidney enzyme was thus compared to the purified enzyme from the same tissue by using controlled trypsinolysis in the presence of Na^+ or K^+ in combination with immunoblotting of the proteolytic fragments. Our data indeed indicate that the two enzyme preparations compare well with respect to the exposure or occlusion of specific tryptic bonds in the two ionic conditions and we might thus conclude that the main topological characteristics of the enzyme are conserved during purification. The only segment of the alpha-subunit which potentially might be sensitive to detergent treatment is the NH_2-terminal. Indeed a cleavage of a 3 kDa fragment occurs in both the Na^+- and the K^+-form of the alpha-subunit from the purified enzyme or from the microsomal enzyme treated with low detergent concentrations while under the same experimental conditions this cleavage only occurs in the Na^+ form of the alpha-subunit from microsomal fractions free of detergents (Fig. 6). The functional consequences of this modification can actually not be estimated. Possibly, it could mean that the

purified enzyme preparation of amphibian source, despite its high specific enzyme activity, has lost some regulatory characteristics, inherent to the N-terminus of the microsomal enzyme. It has been reported that in the mammalian enzyme, the 3 kDa cleavage occurs in the Na^+- and the K^+-form but is more sensitive in the Na^+-form [11]. Since this is true even with purified preparations, it might indicate that the mammalian enzyme is even less sensitive to structural modifications by detergents than the amphibian enzyme.

(3) Antigenic determinants of the alpha-subunit from amphibian ($Na^+ + K^+$)-ATPase

Tryptic cleavage of the alpha-subunit combined with Western blotting and immunooverlay of the samples provided us with valuable information on the antigenic determinants of various domains of the alpha-subunit. Our data suggest that the N-terminal domain (e.g. the 40 kDa fragment) contains more antigenic sites than the C-terminal domain (e.g. the 56 kDa fragment). These data are in good agreement with a recent report by Farley et al. [25] who determined three main antigenic domains on the alpha-subunit of dog kidney, all located within the amino terminal half of the polypeptide. Since their antibodies had been produced against denatured purified alpha-subunit, it seems that the NH_2-terminal has a higher inherent antigenicity compared to the C-terminal with respect to continuous as well as discontinuous epitopes.

The present study provides in addition further evidence for the specificity of our anti alpha-serum Indeed, the good correspondence of the tryptic pattern of the alpha-subunit from the purified toad kidney enzyme with the one from other highly purified preparations together with the good correlation existing between the disappearance of the material in the alpha-subunit gel region and the loss of ($Na^+ + K^+$)-ATPase activity upon trypsinolysis suggest that the antigen, namely the polypeptide of 96 kDa which had been used to prepare the anti-alpha-serum [12], displays a high degree of homogeneity.

In conclusion, the present study enabled us to demonstrate the similarity of the overall structural organization of the alpha-subunit in different ($Na^+ + K^+$)-ATPase preparations. In addition, we could define in this study major antigenic domains on the alpha-subunit. This information was a prerequisite for the assessment of structural and functional maturation processes of the ($Na^+ + K^+$)-ATPase in the intact cell [20] and has been of critical importance for the successful molecular cloning of the amphibian alpha-subunit, where our antisera were used for screening of a cDNA expression library [26].

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References

1 Jørgensen, P L (1986) Kidney Int 29, 10–20
2 Kaplan, J H (1985) Annu. Rev Physiol 47, 535–544
3 Brotherus, J R., Jacobsen, L and Jørgensen, P L (1983) Biochim Biophys. Acta 731, 290–303
4 Jørgensen, P L (1974) Biochim Biophys Acta 356, 36–52
5 Jørgensen, P L., Karlish, S J D and Gitler, C (1982) J Biol Chem 257, 7435–7442
6 Kawakami, K, Noguchi, S, Noda, M, Takahashi, H, Ohta, T, Kawamura, M, Nojima, H, Nagano, K, Hirose, T., Inayama, S, Hayashida, H Miyata, T and Numa, S (1985) Nature 316, 733–736
7 Shull, G E, Schwartz, A and Lingrel, J B (1985) Nature 316, 691–695
8 Anner, B.M (1985) Biochim Biophys Acta 822, 319–334
9 Anner, B M (1985) Biochim Biophys Acta 832, 335–353
10 Jørgensen, P L (1975) Biochim Biophys Acta 401, 399–415
11 Jørgensen, P L (1977) Biochim Biophys Acta 466, 97–108
12 Girardet, M, Geering, K, Frantes, J M, Geser, D, Rossier, B C, Kraehenbuhl, J P and Bron, C (1981) Biochemistry 20, 6684–6691
13 Lowry, O H, Rosebrough, N.J, Farr, A L and Randall, R J (1951) J. Biol Chem. 193, 265–275
14 Geering, K and Rossier, B C (1979) Biochim Biophys Acta 566, 157–170
15 Hokin, L E, Dahl, J L, Deupree, J D, Dixon, J F, Hackney, J F and Perdue, J F. (1973) J Biol Chem 248, 2593–2605
16 Smith, D E, and Fisher, P A (1984) J Cell Biol. 99, 20–28

17 Towbin, H , Staehelin, T. and Gordon, J. (1979) Proc Natl Acad Sci U.S A 76, 4350–4354
18 Burnette, W.N (1981) Anal. Biochem. 112, 195–203
19 Muilerman, H G , Ter Hart, H.G J and Van Dijk, W (1982) Anal Biochem 120, 46–51
20 Geering, K , Kraehenbuhl, J.P and Rossier, B C (1987) J Cell Biol., in press
21 Castro, J and Farley, R A. (1979) J. Biol Chem 254, 2221–2228
22 Lo, T N , and Titus, E O (1978) J Biol Chem 253, 4432–4438
23 Jørgensen, P L and Farley, R A (1986) Meth Enzymol , in the press
24 Zurini, M , Krebs, J , Penniston, J.T and Carafoli, E (1984) J Biol Chem 259, 618–627
25 Farley, R A , Ochoa, G.T. and Kudrow, A. (1986) Am. J Physiol 250, C896–C906
26 Verrey, F., Schaerer, E , Zoerkler, P , Paccolat, M P , Geering, K , Kraehenbuhl, J P and Rossier, B C (1987) J Cell Biol. 104, 1231–1237