LIGAND-DEPENDENT TRYPTIC INACTIVATION OF THE OUABAIN SENSITIVITY OF ADP-ATP EXCHANGE CATALYZED BY CANINE RENAL Na^+,K^+-ATPase

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Summary: Trypsin treatment of purified canine renal Na^+,K^+-ATPase preparations causes loss of Na^+,K^+-ATPase activity with production of tryptic fragments as described previously (Jorgensen, P. L. (1975) Biochim. Biophys. Acta 401, 399-415). With 0.08-0.1 M Na^+ or K^+ present, the Na^+-dependent, ouabain-sensitive ADP-ATP exchange activity is also inactivated by trypsin. Addition of 5 mM ATP to K^+-containing digestion mixtures results in protection of ADP-ATP exchange activity with an unexpected loss of ouabain sensitivity. Addition of MgATP to Na^+-containing digestion mixtures also protects exchange activity, and in this case ouabain-sensitivity is also lost, regardless of the presence or absence of MgATP. In all cases where ouabain-insensitive exchange activity is generated, the 115,000 dalton α subunit is degraded to an 87,000 dalton fragment. When loss of activity occurs without development of ouabain-insensitive exchange activity the α subunit is cleaved to 52,000 and 67,000 dalton fragments.

Introduction: Canine kidney Na^+,K^+-ATPase preparations resemble others (1-4) in showing two major bands on SDS-polyacrylamide gels with molecular weights of approximately 115,000 and 70,000 (5). The purified enzyme probably occurs in canine kidney as a tetramer ($\alpha_2\beta_2$), the integrity of which is required for overall Na^+,K^+-ATPase activity (2, 4-7). This preparation also catalyzes, among other "partial" activities, a ouabain-sensitive, Na^+-dependent ADP-ATP exchange activity, which probably does not require intact tetrameric structure to remain functional (5,8,9). The present communication presents preliminary evidence indicating that even proteolytically cleaved α subunits can catalyze ADP-ATP exchange activity. In addition, the present results show that trypsin cleavage to yield certain specific products results in a preparation with ouabain-insensitive exchange activity. This finding begins to define in a preliminary way some of the structural bases of subunit function in purified Na^+,K^+-ATPase preparations.

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Methods: The preparation of purified Na^+,K^+-ATPase by a slight modification of the method of Jorgensen (4) has been previously described (9). Methods for measuring Na^+,K^+-ATPase and ADP-ATP exchange activities have been reported elsewhere (8).

Trypsin treatment was carried out at 37° with an enzyme:trypsin ratio of 20 and 1.8 mg Na^+,K^+-ATPase protein per ml. The reaction was terminated by adding a 3-fold excess by weight of soybean trypsin inhibitor (1 mg inactivates 2.18 mg trypsin). Aliquots were then taken for measuring exchange activity or for SDS-polyacrylamide gel electrophoresis by the method of Fairbanks et al. using 7% gels (10). Gels were stained with Coomassie blue.

Results: As previously reported by Jorgensen for rabbit kidney enzyme (11), trypsin treatment inactivates Na^+,K^+-ATPase activity in a fashion dependent on whether Na^+ or K^+ are present in the digestion medium (results not shown): 1) When proteolysis is carried out in 0.1 M K^+, loss of enzyme activity is linear with time when plotted semilogarithmically, suggesting a first-order process. 2) When 0.1 M Na^+ is present during digestion, biphasic semilogarithmic plots are obtained. 3) Addition of ATP to K^+-containing digest, or MgATP to Na^+-containing digests, causes the kinetics of loss in each case to change to the pattern typical of the other alkali metal cation, e.g., addition of ATP to K^+-containing digestions causes the linear semilogarithmic plot to become biphasic (as though Na^+ were the cation present). 4) Jorgensen correlated these kinetics of inactivation with changes in the products of tryptic digestion depending on whether Na^+ or K^+ was present and concluded that there are two conformations of the Na^+,K^+-ATPase,a "K^+-form" and a "Na-form". Our results with canine kidney enzyme qualitatively resemble these findings and will be discussed below.

When the Na^+-dependent, ouabain-sensitive ADP-ATP exchange activity is measured following trypsin treatment, a markedly different behavior is seen. Fig. 1 shown a typical semilogarithmic plot of exchange activity as a function of time of digestion with trypsin in the presence of 84 mM KCl. Loss of exchange activity is non-linear in such plots (in contrast with loss of ATPase activity) and the activity surviving at each time interval remains ouabain-sensitive (open symbols). Addition of 5 mM ATP, however, protects the exchange activity against tryptic inactivation (filled symbols). Unexpectedly, the exchange activity surviving under these conditions becomes progressively ouabain-insensitive as the digestion proceeds.

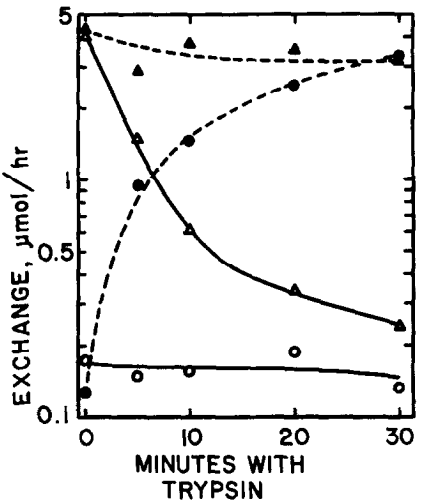

Fig. 1. Effect of trypsin treatment in 83 mM KCl on the ADP-ATP exchange activity of canine kidney Na^+,K^+-ATPase. Open symbols, KCl alone; filled symbols, KCl + 5 mM ATP. Assays of activity were carried out without (triangles) and with (circles) 1 mM ouabain present. Trypsin treatment is described under Methods. Exchange activity is plotted on a logarithmic scale. Concentrations during assay: Na^+, 12.5 mM; K^+, 3.2 mM. Other experiments assayed at 125 mM Na^+ produced similar results. Values shown are the means of triplicate determinations.

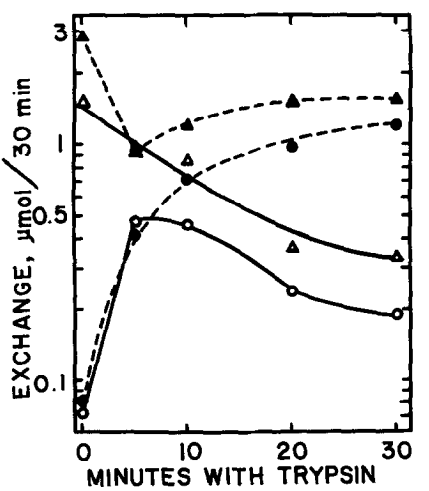

Fig. 2. Effect of trypsin treatment in 0.1 M NaCl on ADP-ATP exchange activity. Open symbols, NaCl alone; filled symbols, NaCl + 5 mM ATP + 3 mM $MgCl_2$. Assays of activity were carried out without (triangles) and with (circles) 1 mM ouabain present. Concentration of Na^+ during assay was 20 mM. Addition of 20 mM K^+ did not qualitatively affect the results. Values are the mean of duplicate determinations.

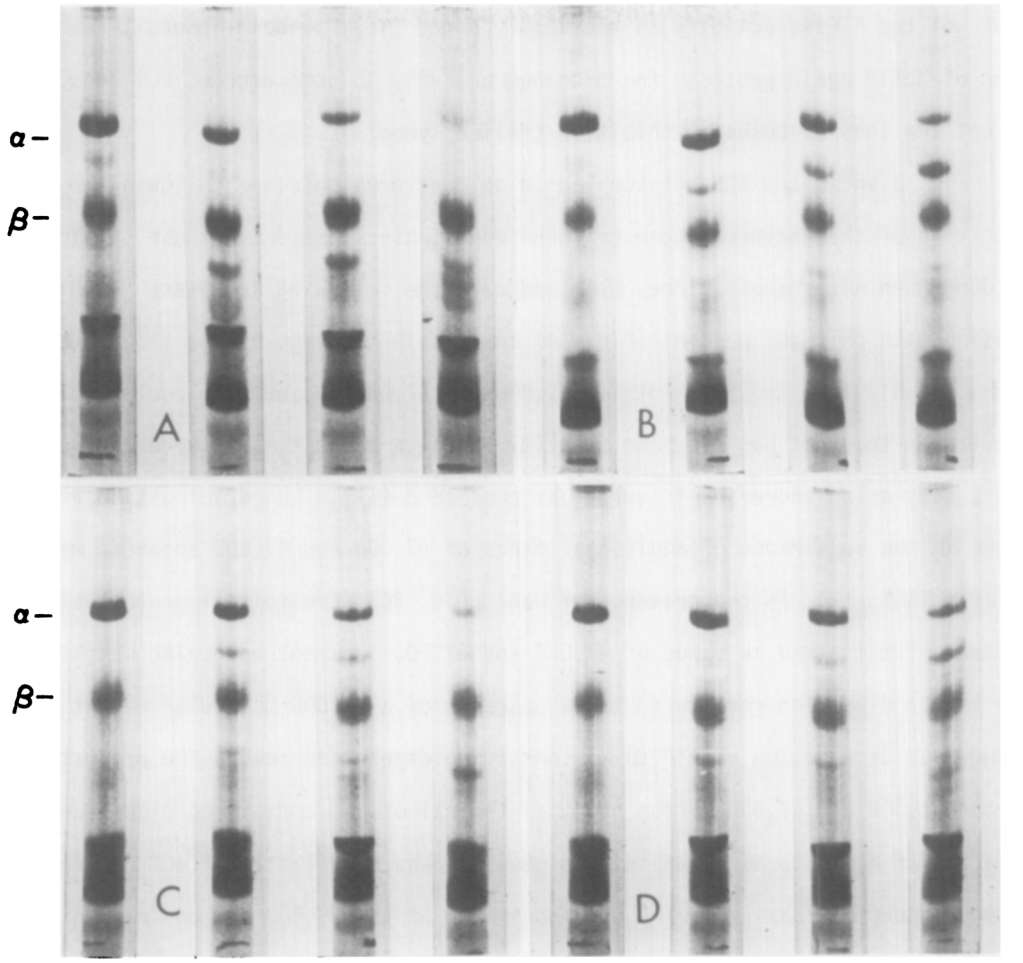

Fig. 3. Products of tryptic digestion as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Times of digestion in each panel are 0, 5, 10 and 30 min (left to right). The two heavy bands at the bottom of each gel are trypsin and soybean trypsin inhibitor. The tracking dye position is marked by India ink. A, 0.1 M KCl present during trypsin treatment; B, 0.1 M KCl + 5 mM ATP; C, 0.1 M NaCl; D, 0.1 M NaCl + 5 mM ATP + 3 mM $MgCl_2$. Gel concentration, 7%.

When digestion is carried out in a similar concentration of NaCl, results such as those of Fig. 2 are obtained. In this case, when Na^+ is present alone, non-linear inactivation plots are also obtained (as with K^+), but now the inactivation is accompanied by loss of ouabain sensitivity. These results suggest that cleavage of the "Na^+-form" of the enzyme renders the exchange activity

(but not the ATPase activity as previously noted (11)) ouabain-insensitive. Addition of MgATP again protects the exchange activity to some degree, but does not affect the loss of ouabain inhibition (filled symbols).

Fig. 3 shows the SDS-polyacrylamide gel patterns obtained following tryptic digestion of the enzyme. These patterns qualitatively resemble those obtained by Jorgensen with rabbit kidney (11) and have the following features: 1) Digestion with only KCl present (Fig. 3A) results in cleavage of the 115,000 dalton α subunit into two fragments of 52,000 and 67,000 apparent molecular weight. The latter fragment has the same mobility as the β subunit, and therefore appears only as an increased intensity of that band (11). Longer digestion results in the appearance of additional bands of 47,000 and 42,000 apparent molecular weight. 2) In the presence of NaCl (Fig. 3C), the initial proteolysis products run instead as bands of 87,000 and 47,000 apparent molecular weight and longer digestion results in the appearance of a 42,000 molecular weight fragment. 3) Addition of ATP to K^+-containing digestions causes the production of the 87,000 and 47,000 dalton products typical of Na^+-containing digestions. Addition of MgATP to Na^+-containing digestions has very little effect on the pattern except perhaps to delay the appearance of the 87,000 dalton fragment. In each case, the cleavage to produce 87,000 dalton fragments results in retention of ADP-ATP exchange activity with loss of its ouabain-sensitivity. Cleavage in K^+ alone to form 52,000 and 67,000 molecular weight fragments causes loss of exchange activity with the surviving fraction remaining ouabain-sensitive.

Discussion: Trypic inactivation of Na^+,K^+-ATPase activity does not necessarily result in loss of ADP-ATP exchange activity. As shown by Jorgensen (11) and confirmed in our laboratory, both the "K^+-form" and "Na^+-form" of the enzyme are progressively inactivated by trypsin and the effect of ATP or MgATP is merely to alter the linearity of semilogarithmic plots of activity versus time and the nature of the polypeptide fragments obtained. Measurement of the ADP-ATP exchange activity reveals, however, that the loss of exchange activity

is markedly diminished by ATP, suggesting that the amount of structural integrity required for this activity is much less than for overall ATPase activity. One might hypothesize that a single cleavage on the $\alpha_2\beta_2$ tetramer is sufficient to incapacitate it without serious effect on the initial phosphorylation site involved in the exchange reaction.

More surprising than this proteolytic discrimination between the two activities is the apparent tendency for the "Na-form" of the enzyme to lose the ouabain-sensitivity of its exchange function when cleaved by trypsin. This ouabain-insensitive exchange seems to be catalyzed by the 87,000 dalton fragment since the appearance of that band coincides with both enhanced survival of exchange activity and loss of its ouabain-sensitivity.

There are several possible ways such a result might arise: 1) Cleavage to the 87,000 dalton fragment may destroy the ouabain binding site. 2) Ouabain binding may remain unaffected, but occur on the smaller cleavage product and thereby be unable to "communicate" with the active site to inhibit it. 3) Ouabain binding may remain intact and involve the 87,000 dalton fragment, but be unable to induce and stabilize an inactive conformation of the enzyme. Other possibilities can no doubt be conceived.

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