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TRYPTIC INACTIVATION OF THE OUABAIN BINDING SITE OF CANINE KIDNEY Na^+,K^+-ATPase AND ITS EFFECT ON CATALYTIC FUNCTION

Charles G. Winter

Department of Biochemistry
University of Arkansas College of Medicine
Little Rock, Arkansas 72201

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Summary: Trypsin treatment of the purified Na^+,K^+-ATPase from canine renal outer medulla causes loss of ADP-ATP exchange activity when digestion takes place in 0.1 M KCl. Activity surviving this treatment remains inhibitable by ouabain. Addition of ATP to such digestion mixtures stabilizes the Na^+,K^+-ATPase in a different conformation (Na^+-form). Under these conditions ADP-ATP exchange activity is protected, and becomes ouabain-insensitive. Quantitative analysis of the cleavage products and rates of loss of ouabain binding and exchange activity suggest that catalytically inactive trypsinolysis products can bind ouabain, and that the 85,000 dalton fragment associated with ouabain-insensitive ADP-ATP exchange activity cannot bind ouabain. Cleavage to produce the 85,000 dalton fragment therefore destroys the ouabain binding site.

Introduction: The Na^+,K^+-ATPase purified from canine kidney outer medulla is an $\alpha_2\beta_2$ tetramer, with "catalytic" (α) subunits of about 100,000 molecular weight and glycoprotein (β) subunits of 55-70,000 molecular weight (1-3). Disruption of this tetramer to yield $\alpha\beta$ dimers inactivates the Na^+,K^+-ATPase activity, but its Na^+-dependent, ouabain-sensitive ADP-ATP exchange activity survives this treatment (3-6), indicating that α subunits can carry out ADP-ATP exchange without the need for intact tetrameric structure.

Controlled tryptic digestion also inactivates the Na^+,K^+-ATPase, and the site of initial cleavage of the α subunit depends on the nature of the ligands present during digestion (7-11). The glycoprotein subunit is trypsin-resistant. Based on these results, Jorgensen postulated two trypsin-sensitive conformations of the Na^+,K^+-ATPase, a Na^+-form and a K^+-form (8). Previous studies of erythrocyte Na^+,K^+-ATPase by Giotta suggested that the site of tryptic attack is on the cytoplasmic surface of the membrane (7).

Cleavage of the α subunit by trypsin also results in loss of both ADP-ATP exchange activity (10) and the ability to be phosphorylated by [γ-^{32}P] ATP (9,11).

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The Na^+-form of the enzyme loses exchange activity much more slowly than overall ATPase activity. Under these conditions, both conversion of the phosphorylated enzyme to an ADP-insensitive form and subsequent K^+-activated dephosphorylation are impaired (11). A surprising additional finding is the fact that the surviving ADP-ATP exchange activity becomes progressively ouabain-insensitive (10).

Previous studies did not allow us to propose a mechanism for loss of ouabain sensitivity. We could not decide whether the ouabain binding site is destroyed or whether ouabain binds normally but fails to produce its inhibitory action on the enzyme. Earlier studies of Na^+,K^+-ATPase from beef brain indicated that ouabain binding is unaffected by trypsin treatment (12), but ligands known to affect enzyme conformation were not added to the digestion mixtures.

We have therefore made quantitative measurements of the effect of trypsin treatment on the ADP-ATP exchange activity, ouabain binding and the nature and amounts of polypeptide products of Na^+,K^+-ATPase preparations in an attempt to understand the mechanism by which ouabain sensitivity is lost.

Methods: The purification of Na^+,K^+-ATPase from canine renal outer medulla by a slight modification of Jorgensen's procedure (13) has been described elsewhere (6). Methods for measuring protein, Na^+,K^+-ATPase and ADP-ATP exchange activities are those used previously (5). Binding of [^{3}H] ouabain in the presence of Na^+ and ATP was carried out as published earlier (6), as was sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10). Duplicate gels were scanned at 580 nm on an ISCO Model 1310 Gel Scanner, and the area of resulting peaks was determined by planimetry.

Tryptic digestion of the purified enzyme was carried out at 37° at an ATPase: trypsin ratio of 20 and 2 mg Na^+,K^+-ATPase protein per ml. The trypsin used was Sigma Type XI. The reaction was terminated at intervals with a 3-fold excess of soybean trypsin inhibitor (1 mg inactivated 1.5-2.2 mg trypsin, depending on the batch used). The samples were then placed in ice and distributed into tubes as necessary for assaying ADP-ATP exchange or [^{3}H] ouabain binding or for preparation for gel electrophoresis.

Results: The ADP-ATP exchange activity catalyzed by purified canine kidney Na^+-K^+-ATPase is progressively inactivated by the action of trypsin on the enzyme, either in the K^+-form or the Na^+-form. During inactivation of the K^+-form, the activity surviving at all times of treatment remains ouabain-inhibitable. An example of this phenomenon is shown in Fig. 1 (dashed lines). Addition of ATP to K^+-containing digestion mixtures causes the enzyme to assume the Na^+-form

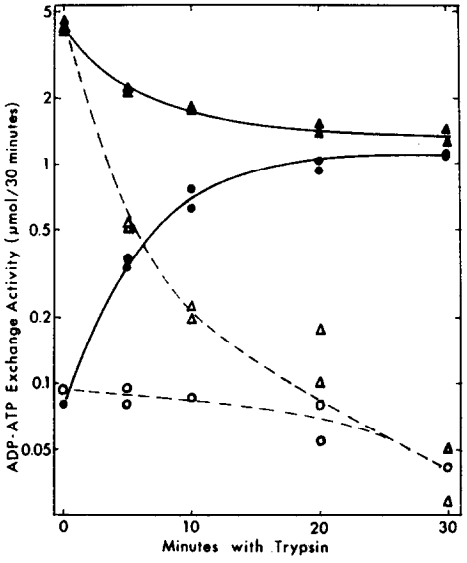

Fig. 1. Effect of trypsin on ADP-ATP exchange and on its sensitivity to inhibition by ouabain. Enzyme suspended in 0.04 M Tris, pH 7.4/0.1 M KCl was digested with trypsin as described under Methods. Aliquots were removed at the times indicated into soybean trypsin inhibitor, then assayed for ADP-ATP exchange activity in the presence or absence of 10^{-3}M ouabain. Open symbols, KCl alone; filled symbols, KCl + 5 mM ATP (Tris salt). Triangles, assayed without ouabain present; circles, assayed with ouabain.

8,10). Under these conditions, the exchange activity is partially protected against tryptic inactivation, but the activity surviving becomes progressively ouabain-insensitive (Fig. 1, solid lines).

Tryptic digestion of the Na^+-form of the enzyme followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis reveals the presence of a new species of about 85,000 molecular weight (Fig. 2). As previously reported, this species appears at the expense of the α subunit (7,8), but not in stoichiometric amounts. Careful inspection of the gels reveals the progressive accumulation of a heterogeneous mixture of products moving between the β subunit and the tracking dye.

Figure 3 illustrates the effect of trypsin on the equilibrium binding of ouabain by the enzyme. In this particular experiment, K^+ and ATP were present during digestion, but similar results are obtained when tryptic digestion is carried out in the presence of KCl alone (Table 1).

The results indicate that ouabain binding is progressively lost after trypsin treatment, irrespective of the conformation of the enzyme. The slight drop in

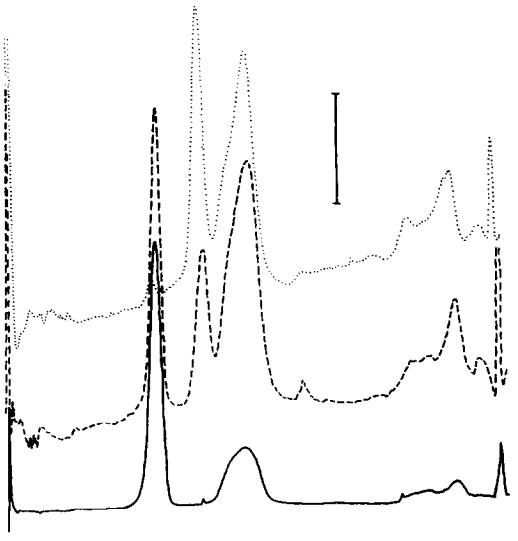

Fig. 2. Products of tryptic digestion of the Na^+-form of the enzyme as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Enzyme was digested in the presence of 0.1 M KCl and 5 mM ATP as in Fig. 1, and as described in Methods. Aliquots were subjected to electrophoresis and the gels scanned at 580 nm. Digestion was for 30 secs (solid line), 10 min (dashed line) and 30 min (dotted line). The vertical bar represents 0.4 absorbance units (30 sec sample) and 0.1 absorbance units (10 and 30 min samples). The sharp vertical deflections at the left and right of each tracing are caused by the top of the gel and an India ink spot marking the tracking dye, respectively. The calculated molecular weight for the α-subunit is 111,300, for the β subunit 65,100 and for the product peak between them 84,800, in reasonable agreement with previously published values (3,10). The measured mass ratio of α to β is 1.73 ± 0.28 SD (n=4). The expected mass ratio for an $\alpha_2\beta_2$ tetramer is 1.71 based on these molecular weights.

observed K_d is probably not significant, since the ATPase activity of the control enzyme at low ouabain concentrations probably influences the slope of the plot. This assumption is supported by the fact that control binding assays not supplemented with ATP as described in Fig. 3 yielded K_d values of 1000-1200 nM, with little change in apparent binding capacity. Taken together, the results therefore indicate that ouabain binding capacity is lost by the trypsin-treated enzyme at a rate independent of enzyme conformation.

Table 2 compares for the same digestion mixture the relative rates of appearance of ouabain-insensitive ADP-ATP exchange and the 85,000 dalton fragment, as well as the disappearance of ouabain-sensitive exchange, α subunit and ouabain binding.

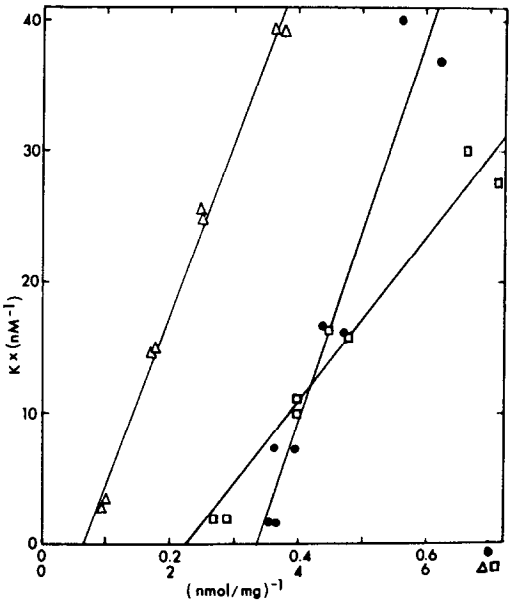

Fig. 3. Effect of trypsin treatment on [^{3}H] ouabain binding by the Na^+,K^+-ATPase. Enzyme was treated with trypsin in the presence of KCl and ATP and [^{3}H] ouabain binding measured as described under Methods. Filled circles, untreated control; triangles 10 min with trypsin; squares 30 min with trypsin. Control samples were supplemented with an additional 5 mM ATP twice during the 30-min incubation to ensure the presence of sufficient substrate at low ouabain concentrations. Scaling factor (K): 10^4 for the control samples; 10^3 for the 10 and 30 min samples. Data are plotted as recommended by Nimmo and coworkers (14). The lines are fitted by linear regression analysis.

Table 1

Effect of Trypsin Treatment on [^{3}H] Ouabain Binding by the Purified Na^+,K^+-ATPase

Enzyme was treated with trypsin and ouabain binding measured as described under Methods. Binding capacities and dissociation constants were obtained by linear regression analysis as in Fig. 3.

Time of treatment (min)	Ligands present	Binding Capacity (nmol/mg)	K_d (nM)
0	-	2.86	189
10	K^+	1.56, 1.67	61,47
30		0.25, 0.27	75,108
10	K^+ + ATP	1.52, 1.64	63,35
30		0.52, 0.42	58,46

Table 2

Effect of Trypsin on Some Characteristics of the Na^+-form of Na^+,K^+-ATPase

Enzyme was digested with trypsin in the presence of K^+ and ATP for the time indicated as described under Methods. Aliquots of the reaction mixture were removed into soybean trypsin inhibitor and then assayed for ADP-ATP exchange, [^{3}H] ouabain binding and α subunit breakdown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All results shown were obtained from the same digestion mixture, hence are directly comparable. Results are expressed as fraction of original activity or amount.

Time of Digestion (min)	Ouabain-sensitive ADP-ATP exchange[a]	α subunits[b]	Ouabain insensitive ADP-ATP exchange[c]	85,000 dalton fragment[b]	Total ADP-ATP exchange	Ouabain binding Capacity[d]
0.5	0.98	0.92	0.021	0	1.00	0.96
10	0.25	0.20	0.15	0.13	0.40	0.59
30	0.055	0.017	0.24	0.28	0.30	0.15

[a] Control activity: 4.3 μmol/30 min.

[b] Fraction of area of α peak for untreated enzyme (average of duplicates).

[c] Fraction of original ouabain-sensitive activity rendered ouabain-insensitive.

[d] Control activity: 2.78 nmol/mg. Using the molecular weights determined for Fig.2, the stoichiometry of ouabain binding is 0.98 mol/mol enzyme tetramer.

It is clear that the appearance of ouabain-insensitive exchange activity agrees quantitatively with the production of the 85,000 dalton fragment, while loss of ouabain-sensitive exchange correlates with loss of intact α subunits. Loss of ouabain binding, however, lags significantly behind the cleavage of α subunits and the loss of ouabain-sensitive exchange.

Discussion: Previous work has led to the conclusion that ouabain binding to the Na^+,K^+-ATPase is unaffected by trypsin treatment of the enzyme. The present study indicates that this is not so. Digestion of both the K^+-form and the Na^+-form result in loss of binding capacity, with little apparent effect on the affinity of the binding site of the surviving binding species for ouabain.

It is not absolutely clear why the ADP-ATP exchange activity becomes ouabain-insensitive when the Na^+-form of the enzyme is cleaved by trypsin. However, in the early stages of digestion (through 10 min, Table 2), total exchange activity

(irrespective of ouabain sensitivity) disappears faster than does ouabain binding. This must mean that there are enzymatically inactive species produced that are still structurally intact at the ouabain binding site. This is consistent with the fact that no significant amount of polypeptide is released from the membrane until the extent of trypsin cleavage exceeds 80% (7,8). That there are multiple breaks produced in the α subunit can easily be seen by summing the remaining amounts of α and the 85,000 dalton fragment: after 10 minutes, only 33% of the material is found in discrete peaks.

Ouabain binds to the Na^+,K^+-ATPase at a stoichiometry of 1 ouabain molecule per tetramer unit (Table 2). Since ouabain-binding species are present that fail to catalyze ADP-ATP exchange, it is clear that that the ouabain-insensitive exchange activity exceeds the fraction of ouabain binding surviving prolonged proteolysis. The logical conclusion, therefore, is that the particles catalyzing ouabain-insensitive exchange activity do not bind ouabain. Thus, cleavage to form the 85,000 dalton product must involve the destruction of the ouabain binding site. Cleavage of the K^+-form of the enzyme to produce the 52,000 and 67,000 dalton fragments apparently does not affect this site, since ouabain sensitivity of the exchange activity is retained (10). Subsequent breakdown at additional cleavage sites then produces loss of ouabain binding, but this loss is not detectable enzymatically since catalytically inactive species are involved.

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