The (Na^+,K^+)ATPase exhibits enzymic activity in the absence of the glycoprotein subunit

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Membrane-bound (Na^+,K^+)ATPase from avian nasal salt glands was exposed to limited papain digestion. Such treatment results in the selective removal of the β-subunit rendering the α-subunit still membrane-bound and expressing full enzymic activity. With further exposure to papain the α-chain becomes fragmented into two major polypeptide components. The fragmented membrane-bound catalytic chain is extremely sensitive to detergent treatment and cannot be solubilized in an active state.

(Na^+,K^+)ATPase *Papain digestion* *Detergent solubilization*

1. INTRODUCTION

Animal cells possess in their plasma membranes an ion pump for sodium and potassium. This pump utilizes the free energy of hydrolysis of ATP to extrude Na^+ and take up K^+ in a coupled fashion, ultimately establishing and maintaining an ion concentration gradient across the cell membrane.

The Na^+ and K^+ activated adenosine triphosphatase [(Na^+,K^+)ATPase] or sodium pump has been isolated from the plasma membrane of many tissues: renal medulla of mammalian kidney [1-3]; rectal glands of dogfish [4,5]; eel electroplax [6,7]; an avian nasal salt glands [8]. In all cases the purified enzyme has been shown to be composed of two dissimilar polypeptide chains present in equimolar concentrations. The catalytic subunit, designated α, contains the binding sites for Na^+, K^+, ATP, the phosphorylation site, and the inhibitory binding site for the cardiac digitalis glycosides [9]. The catalytic subunit has M_r 94 000–106 000 [10–12], and spans the plasma membrane.

The glycoprotein chain, designated β, has M_r 41 000–52 000 and is thought to be exposed only to the outer surface of the cell [13]. The function of the β-chain as a component of this molecular assembly is still unknown. All attempts to separate the α and β-chains have resulted in an irreversible loss in enzyme activity. Since this enzyme is an integral membrane protein, it may be that conditions required to dissociate the α-chain from the β-chain (e.g., detergent solubilization) also lead to protein denaturation.

Here, we describe the selective removal of the β-subunit by exposing the membrane-bound enzyme to limited papain digestion. The remaining membrane-bound protein assembly expresses virtually full ATPase activity and is composed of only a fragmented α-chain. The fragmented catalytic chain is extremely sensitive to detergent treatment and cannot be solubilized in an active state.

2. METHODS AND MATERIALS

2.1. *Preparation of (Na^+,K^+)ATPase*

Nasal salt glands were dissected from decapitated terns and sea gulls and immediately frozen on dry ice. After transportation to the laboratory, a microsomal fraction of the salt gland was prepared in a manner virtually identical to that in [8]. Each gland yielded about 1 mg of microsomal protein. The enzyme activity was stable to storage indefinitely as a microsomal fraction at −70°C. The purified enzyme was then obtained as a membrane-bound fraction by extracting the microsomes with

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SDS followed by discontinuous sucrose gradient centrifugation. Each microsomal fraction was pretitrated with SDS as in [8], to determine optimal conditions for detergent extraction. The final pellets were resuspended in 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA and stored at 4°C.

2.2. *Detergent solubilization of the (Na⁺,K⁺)ATPase*

Purified, membrane-bound (Na^+,K^+)ATPase was diluted to 1 mg protein/ml into 25 mM TES (pH 7.5), 1 mM EDTA, 20% glycerol, 0.1 M NaCl. Solubilization was achieved by adding Brij 58 ($C_{16}E_{20}$) to a final concentration of 5 mg/ml, incubating for 30 min at 25°C, and then removing insoluble material by centrifugation at $100000 \times g$ for 60 min. The solubilized enzyme present in the supernatant solution was then chromatographed on a Sepharose CL-4B column (1.5 × 90 cm) equilibrated in 25 mM TES (pH 7.5), 1 mM EDTA, 0.1 M NaCl, 25 μM Brij 58 at 4°C.

2.3. *Papain digestion of membrane-bound (Na⁺,K⁺)ATPase*

Purified membrane-bound (Na^+,K^+)ATPase (500 μg) was suspended in 500 μl buffer (0.15 M NaCl, 5 mM EDTA, 25 mM cysteine, 20 mM Tris–HCl (pH 7.5) at 23–25°C. Papain (500 ng, 2 times crystallized from Worthington) was added and the mixture was allowed to incubate at room temperature (23–25°C) with gentle mixing. At various time intervals, 75 μl aliquots of the mixture were removed and immediately centrifuged at $100000 \times g$ for 15 min in a Beckman airfuge. Supernatant solution (50 μl) was combined with 50 μl 25% SDS, 5% 2-mercaptoethanol and then heated at 100°C for 10 min. The pellet was solubilized in 75 μl 5% SDS, 1% 2-mercaptoethanol and heated as above. These two samples were then analyzed by SDS–polyacrylamide gel electrophoresis. Small aliquots of the whole mixture and the supernatant fraction were also tested for ATPase activity.

2.4. *Detergent treatment of the papain-digested (Na⁺,K⁺)ATPase*

Purified membrane-bound (Na^+,K^+)ATPase (500 μg) were treated with 500 ng papain as described. After 2 h, the sample was centrifuged at $100000 \times g$ for 30 min at 4°C. The pelleted membraneous material was resuspended in 1 ml 3 M glycerol, 1 mM EDTA, 10 mM KCl, 20 mM imidazole buffer (pH 7.5). Small aliquots of a concentrated solution of Brij-58 were added to attain various concentrations. (Na^+,K^+)ATPase activities were followed kinetically by diluting 10 μl of the mixture into an assay medium at 37°C.

2.5. *(Na⁺,K⁺)ATPase assay*

Ouabain-sensitive ($Na,^+K^+$)ATPase activity was measured using the coupled enzyme assay system in [14]. All assays were performed at 37°C in 0.1 M NaCl, 0.02 M KCl, 5 mM MgATP, 1 mM EGTA, 10 mM TES (pH 7.5) with and without 10^{-4} M ouabain in a total volume of 1 ml. The reaction was initiated by the addition of ATPase and was monitored continuously at 340 nm.

2.6. *Other*

The continuous electrophoresis system of [15] was used with minor modification. Gels stained with Coomassie blue were scanned at 565 nm using a Gilford spectrophotometer equipped with a gel scanning accessory.

Protein was determined as in [16] except when interfering substances were present (e.g., glycerol, EDTA, dithiothreitol, Brij 58) and then the assay in [17] was used. In all cases, bovine serum albumin was used to establish a standard protein calibration curve.

Total neutral sugar content was determined by a modified phenol–sulfuric assay [18]. A standard calibration curve was determined using a 1:1 mixture of mannose and galactose.

3. RESULTS

The (Na^+,K^+)ATPase was purified from the microsomes of avian nasal salt glands by SDS extraction as in [8]. This purified membrane-bound enzyme had spec. act. 28–32 $\mu mol.min^{-1}.mg^{-1}$. Analysis by SDS–polyacrylamide gel electrophoresis revealed >95% of the Coomassie blue staining material was the α- and β-chains.

An aqueous suspension of membrane-bound (Na^+,K^+)ATPase was exposed to twice-crystallized papain at an ATPase to papain ratio of 1000:1. With time, the mixture was separated into a membrane fraction and an aqueous soluble fraction by

Table 1

(Na^+,K^+)ATPase activity (μmol.min^{-1}.mg^{-1})

Time point	Total mixture	100 000 × g pellet	100 000 × g supernatant
0 h	30.6	30.0	0
1 h	29.1	29.7	0
2 h	31.2	31.2	0
4 h	29.0	29.0	0
6 h	27.0	26.2	0
24 h	9.2	8.7	0

centrifugation at 100 000 × g. These two fractions were then analyzed for ATPase activity (table 1) and polypeptide patterns by gel electrophoresis (fig. 1). Treatment of the membrane-bound ATPase with papain resulted in a rapid disappearance of the polypeptide chain corresponding to the β-subunit followed by a progressive cleavage of the α-subunit into fragments of 45 kDa and 35 kDa. These fragments remain membrane-bound since they readily sediment at 100 000 × g and virtually nothing was found in the supernatant fraction. A parallel series of gels were also stained for carbohydrate using a periodate-Schiff staining method

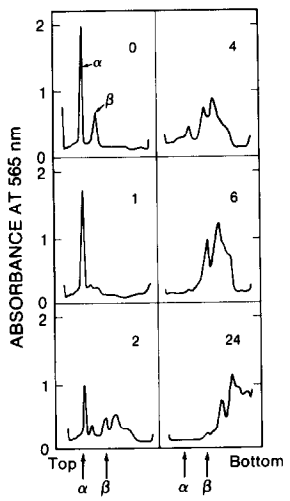

Fig. 1. Papain digestion of the membrane-bound (Na^+,K^+)ATPase. Purified membrane-bound (Na^+,K^+)-ATPase was treated with papain and followed by SDS–polyacrylamide gel electrophoresis. Above are the densitometric scans of gels taken at 0,1,2,4,6 and 24 h papain treatment.

[18]. The native undigested control showed a periodate-Schiff positive band migrating in the position of the β-subunit whereas all digestion intervals were totally periodate-Schiff negative, even when the amount of protein loaded on each gel exceeded to zero time point by 5-fold (not shown).

The ATPase activity associated with these samples at the various time intervals was quite surprising in view of the electrophoretic patterns. Virtually 100% of the initial ATPase activity was retained after 6 h papain exposure even though the β-subunit had been completely removed by digestion and the α-subunit had been fragmented into two polypeptide fragments of 35 kDa and 45 kDa. Furthermore, the (Na^+,K^+)ATPase activity was still 100% ouabain-inhibitible. Not until extensive digestion had occurred (24 h) did the enzyme activity begin to disappear.

Since the electrophoretic analysis does not eliminate the possibility that a fragmented β-subunit may remain associated under non-denaturing conditions; viz., prior to treatment with SDS, the samples were also analyzed for carbohydrate chemically. This data is shown in table 2. After 1 h treatment with papain virtually no carbohydrate was found associated with the membrane fractions which contained the α-subunit and the ATPase activity. Note, sucrose was washed out of the starting material prior to performing this experiment.

The sensitivity of the papain-treated ATPase to detergent-treatment was tested with the nonionic detergent Brij 58. This detergent has been shown to be capable of completely solubilizing the avian ATPase from purified membranes, in fully active form [20]. A 2 h papain digest was chosen and compared to a control undigested sample (fig. 2).

Table 2

nmol neutral carbohydrate

Time point	Total mixture	100 000 × g pellet	100 000 × g supernatant
0	55	50	0
1 h	62	5	50
2 h	55	0	47
4 h	51	0	35
6 h	54	0	49
24 h	50	0	49

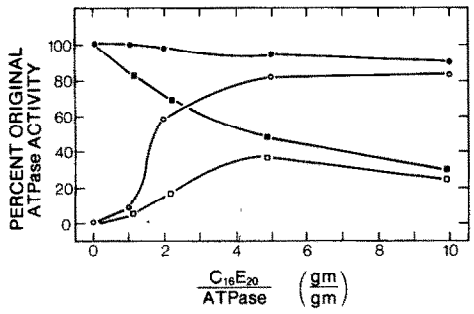

Fig. 2. Detergent solubilization of native membrane-bound (Na^+,K^+)ATPase (o,•) and papain digested (Na^+,K^+)ATPase (□, ■). The ATPase activity present in the whole mixture (•, ■) and in the detergent solubilized fraction (o, □) are plotted as a function of the detergent of ATPase ratio.

Obviously, the papain-treated ATPase was much more sensitive to inactivation by detergent than was the native membrane-bound enzyme. Furthermore, the enzyme activity associated with these detergent–treated papain digests were extremely labile at 37°C and exhibited rapid decay in the assay mixture. The native control enzyme showed stable linear kinetics over the time-course of the assay (5–10 min at 37°C).

Finally, a detergent-solubilized (Na^+,K^+)-ATPase fraction was treated with papain and followed for enzyme activity and polypeptide composition (fig. 3). The solubilized ATPase was obtained by treating the membrane-bound enzyme with Brig 58 at a detergent ATPase 10:1 (w/w) as in section 2. Papain, at 1:1000 (w/w) was totally ineffective at either reducing the ATPase activity or fragmenting the ATPase polypeptides. The addition of Na^+ or K^+ had no effect on these results.

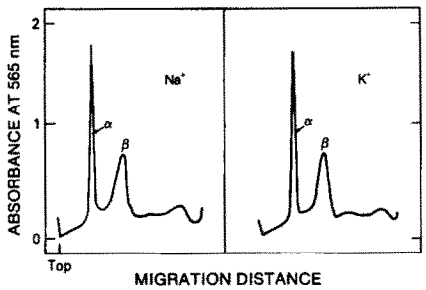

Fig. 3. Papain treatment of detergent-solubilized (Na^+,K^+)ATPase. Brij-solubilized ATPase was exposed to papain for 2 h in the presence of 150 mM NaCl and 150 mM KCl. Aliquots were analyzed by SDS–polyacrylamide gel electrophoresis.

4. DISCUSSION

A major question regarding the (Na^+,K^+)-ATPase which has gone unanswered for many years is what is the function of the glycoprotein chain. A similar unanswered question addresses whether or not the β-chain is actually a component of the pump assembly or simply an extraneous protein which associates tightly to the α-chain during the isolation procedure. The mere fact that the α- and β-chains are present in equimolar amounts in purified membrane preparations which contain only these two subunits, is strong testimony that the β-chain is an integral part of the ion pump [21,22]. Furthermore, solubilization of an active enzymic species from the membrane with the use of various detergents always finds the β-chain to be present tightly complexed with the α-chain in the same detergent micelle [10,11]. However, when strong detergents are used which readily promote dissociation of the α- and β-chains, there is concomitant loss of enzyme activity [23,24].

Several studies [25–27] have examined the effect and fragmentation patterns generated by the action of the proteolytic enzymes trypsin and chymotrypsin on the membrane-bound (Na^+,K^+)-ATPase. With both these proteases, the α-chain is typically cleaved at a minimal number of sites (1 or 3) with an associated loss in ATPase activity. The β-chain, however, remained uncleaved by these two enzymes.

The results reported in this study for papain are quite different than the trypsin results. Exposure of the purified membrane-bound (Na^+,K^+)-ATPase to small amounts of papain resulted in the rapid disappearance of the β-chain followed by a progressive fragmentation of the α-chain into two major fragments of 35 kDa and 45 kDa. The fragmented α-chain remained membrane-bound and retained full capacity to hydrolyze ATP in a ouabain-sensitive manner. This result strongly suggests that the β-chain is not required for the α-chain to express ATP hydrolytic activity or cardiac glycoside sensitivity. Further exposure of the papain fragmented α-chain to detergents capable of solubilizing the native enzyme in active form [20], resulted in a rapid inactivation. This suggests that

an assembly composed of these papain-derived α-chain fragments is required to form the ATP-hydrolyzing unit.

In contrast, papain was found to be without effect on the detergent-solubilized (Na^+,K^+)ATPase. This result is similar to that found for the treatment of Brij 58-solubilized (Na^+,K^+)ATPase with trypsin [20]. Apparently, the large polar head group of the detergent micelle prevents the proteolytic enzyme from finding the susceptible cleavage sites. This also suggests that the β-chain is substantially buried within this head-group region.

Although these results strongly indicate that the β-chain is not required for the expression of ATPase activity, the β-chain may still play a very important role in the function of this protein assembly as an ion pump. Reconstitution of the (Na^+,K^+)ATPase into a lipid vesicle will be required to answer this question more completely. The fact that the β-chain is exposed extracellularly and contains carbohydrate raises the possibility that it may function as a regulatory subunit/membrane-receptor. Obviously, the (Na^+,K^+)ATPase is an important piece of cellular machinery, consuming vasts amounts of cellular energy; it is hard to imagine that it churns away unregulated.

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