Papain digestion of the subunits of (Na,K)-ATPase

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Membrane-bound (Na,K)-ATPases were exposed to limited papain digestion. We could not find the active (Na,K)-ATPase lacking glycoprotein subunit for the enzymes from three different sources (outer medulla of dog kidney, electric organs of *Narke japonica* and larvae of *Artemia salina*). It seemed unlikely that the glycoprotein subunit was selectively removed from (Na,K)-ATPase by papain digestion

(Na^+,K^+)-ATPase *Papain digestion* *Glycoprotein subunit*

1. INTRODUCTION

Plasma membranes of animal cells contain the sodium- and potassium-activated adenosine triphosphatase, (Na,K)-ATPase, responsible for the active transport of Na^+ and K^+ across the cell membrane. All of the (Na,K)-ATPases isolated from different sources so far have been shown to be composed of two kinds of polypeptides: a catalytic subunit (α) of M_r 93000–105000 and a smaller glycoprotein subunit (β) of M_r 32000–38000 [1–3]. The α-subunit contains many important sites for the enzyme, for example, the binding sites for Na^+, K^+, ATP and ouabain and the site for phosphorylation. The function of the β-subunit, however, is still unclear, although the integrity of the β-subunit in the enzyme complex has been shown by the facts that: (a) it copurifies with the α-subunit; (b) antibodies against the β-subunit inhibit (Na,K)-ATPase [4,5]; (c) it can be cross-linked with the α-subunit [6,7]; (d) photoaffinity derivatives of ouabain bind to the β-subunit as well as the α-subunit [8,9]; (e) the molecular size of the unit of (Na,K)-ATPase corresponds to that of the $\alpha\beta$-protomer as revealed by radiation inactivation [10,11] and by gel permeation by high-performance liquid chromatography [12].

Recently, we have reported that the disulfide bond(s) of (Na,K)-ATPase is located within the β-subunit, whose reduction results in the complete loss of enzyme activity [13]. The reduction is, moreover, affected by the addition of Na^+ or K^+ to the medium and not by choline ions [14]. These results clearly indicate that the two subunits are tightly coupled.

However, Freytag [15] has claimed in his article that the β-subunit is not required for the expression of ATPase activity based on the results obtained by limited digestion of avian nasal salt gland enzyme with papain. The resulting enzyme diminishes the β-subunit, leaving the α-subunit still membrane bound and expressing full enzymic activity.

The present results suggest the possibility that the β-subunit is an integral component of (Na,K)-ATPase and that the subunit is not preferentially removed from the membrane by limited digestion with papain.

2. MATERIALS AND METHODS

2.1. *Enzyme preparations*

Enzyme purifications from dog kidney outer

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medulla, nauplii of *Artemia salina* and electric organ of *Narke japonica* were carried out by the methods of Jorgensen [16], Peterson and Hokin [17] and Hayashi and Post [18], respectively. The enzymes obtained were still membrane bound and no further solubilization and purification were performed.

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

Digestion of (Na,K)-ATPase with papain was performed essentially according to Freytag [15]. Purified enzyme was suspended at a protein concentration of 1 mg/ml in a 0.5 ml digestion mixture containing 0.15 M NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol and 20 mM Tris-HCl (pH 7.5). Unless otherwise specified, papain was added at a papain/protein ratio of 1/1000 by wt and the mixture was incubated at 25°C for various periods of time with gentle stirring. At the end of incubation, a 1/10 vol. of 50% perchloric acid was added to inactivate papain. As will be described later, the addition of SDS was not effective in inducing complete inactivation of papain even when the papain concentration was very low. Hence it was essential to add acid prior to the addition of SDS. Papain would otherwise cleave SDS-denatured (Na,K)-ATPase.

2.3. *(Na,K)-ATPase assay*

Ouabain-sensitive (Na,K)-ATPase activity was measured in 0.5 ml medium containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5) and 5–7 μg (Na,K)-ATPase. The reaction was started by adding 0.05 ml of 30 mM ATP. For termination of the reaction, 4.5 ml ammonium molybdate-sulfate solution was added. Inorganic phosphate was assayed by the method of Fiske and SubbaRow [19].

2.4. *Other procedures*

SDS gel electrophoresis was carried out according to Laemmli [20]. Protein concentration was determined by the method of Lowry et al. [21] using bovine albumin as the standard. Correction by quantitative amino acid analysis was not performed. Papain was purchased from Worthington (twice crystallized). All other chemicals were reagent grade or better quality.

3. RESULTS

Purified (Na,K)-ATPase from dog kidney at 1 mg/ml was digested with 1 μg/ml papain at 25°C. As mentioned by Freytag, the activity of the resulting (Na,K)-ATPase remained nearly complete as shown in fig.1A. However, when the papain concentration was increased to 10 μg/ml, the residual activity was decreased as incubation time elapsed. In fig.1B, the amounts of papain added were changed while the incubation period was kept constant for either 1 or 6 h. As expected from fig.1A, the activities of the 6 h incubated sample progressively decreased with increased papain additions. To determine the positions where papain cleaved, the samples were then analyzed by SDS gel electrophoresis.

Since we were not sure whether papain could be inactivated by the addition of 2% SDS, which had been routinely used in our laboratory to solubilize samples for electrophoresis, the conditions for termination of the papain reaction were examined.

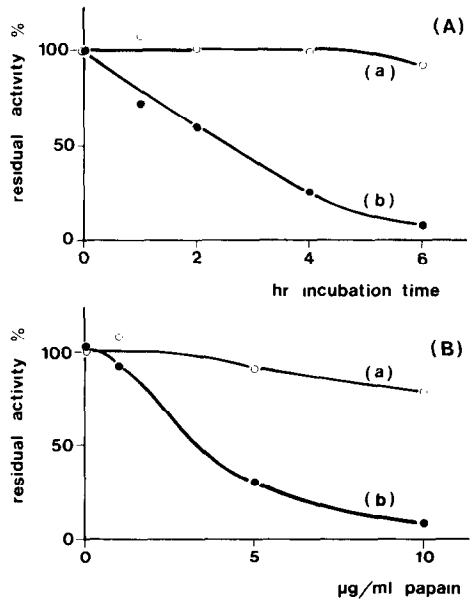

Fig.1. Residual activity of papain-digested (Na,K)-ATPase. Membrane-bound (Na,K)-ATPase was digested with papain at 25°C. (A) Digestion period was varied at constant papain concentrations of 1 and 10 μg/ml for a and b, respectively. (B) Papain concentration was changed while the digestion periods were kept constant for either 1 (a) or 6 h (b).

(Na,K)-ATPase at 1 mg/ml was treated with 1 μg/ml papain at 25°C for 1 h. The resulting enzyme was then centrifuged for 15 min in a Beckman airfuge. The pellet was suspended in the digestion mixture without papain and then solubilized with SDS in 3 different ways: (a) 5% perchloric acid was added to the pellet suspension prior to the addition of 2% SDS, and hence any trace amount of papain in the pellet should be inactivated in this case; (b) the pellet suspension was heated at 100°C for 15 min immediately after the addition of 2% SDS; (c) the pellet suspension was treated with 2% SDS as usual. The second procedure was identical to that described by Freytag. SDS gel electrophoretograms of these samples are shown in fig.2. On all the gels of papain untreated samples, the bands of the α- and β-subunits were clearly seen. The splitting of the α-band seen on the gel of the heated sample has already been reported [22]. On the other hand, there were marked differences in the band patterns among the gels of papain-treated samples. Neither of the bands of the α- or β-subunit was recognized in the sample solubilized by method c. In the heated sample (b), the band of the α-subunit was not present but the band located at the position of the β-subunit was evident. The disappearance of these bands probably resulted from the digestion of SDS-denatured subunits with a trace amount of papain left in the pellet after centrifugation, because both α- and β-subunit bands were clearly identified on the gel for the sample treated by method a where papain was inactivated with acid prior to the addition of SDS. An extremely low concentration of papain (5 ng/ml) could, in fact, digest 0.5 mg/ml (Na,K)-ATPase completely in the presence of 1% SDS (not shown). Therefore, it

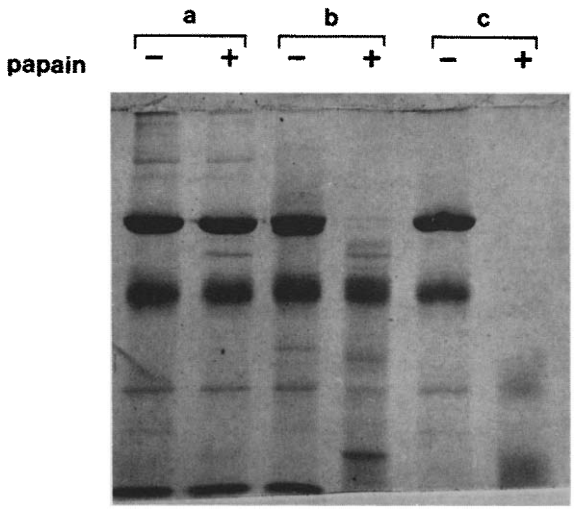

Fig.2. Solubilization of papain-digested (Na,K)-ATPase with SDS. For conditions of solubilization, see text. Electrophoretograms of control and papain-digested samples: − and +, respectively.

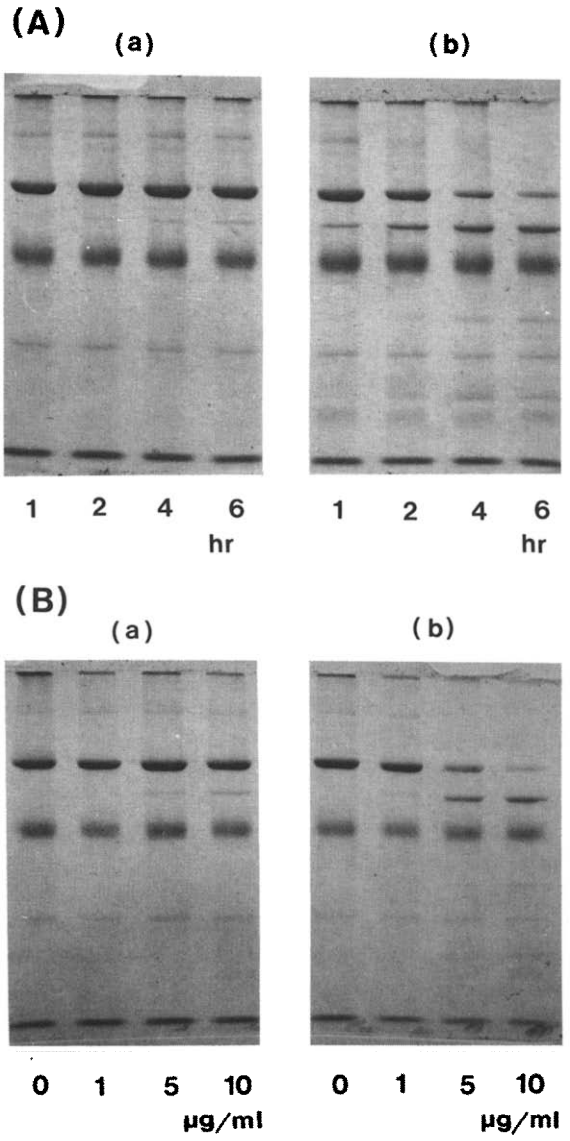

Fig.3. Electrophoretograms of papain-digested (Na,K)-ATPase. Conditions for digestion are the same as for fig.1. (A,B) and (a,b) correspond to those used in fig.1.

was necessary to inactivate papain with acid before the addition of SDS to the digestion mixture. Thereafter papain digestion was terminated by the addition of 5% perchloric acid and the samples were then solubilized for electrophoresis after centrifugation at 2500 rpm for 10 min. Ultracentrifugation with a Beckman airfuge was no longer utilized.

The papain-digested samples whose residual activities are shown in fig.1 were then analyzed by gel electrophoresis. The results are shown in fig.3. The more the residual activities decreased, the less evident was the band of the α-subunit, while the β-subunit bands were not significantly changed.

There was a possibility that the band located at the β-subunit position was due not only to the β-subunit itself but also to a fragment of the α-subunit especially when the digestion with papain progressed. We ruled out that possibility as follows.

Omori et al. [23] have reported that the mobility of the β-subunit on the SDS gel is affected by treatment with sialidase and that the subunit treated with sialidase moves faster than an untreated one. If the band at the position of the β-subunit were due to the β-subunit alone, the band should, therefore, have moved faster to the front when the sample was treated with sialidase. Papain-digested (Na,K)-ATPase was then further treated with sialidase and electrophoresed. The result is shown in fig.4. The band at the β-subunit position moved far to the front while others stayed at their original position. These results indicated that the band was due to the β-subunit itself. We could, however, find a faint band on the gel for the sialidase-treated sample at the positions where the β-subunit was originally located. This was probably due to the papain-digested fragment of the α-subunit, because no such band was seen on the gel of papain-undigested and sialidase-treated samples. Even if this were taken into consideration, we could conclude that the main protein composing the band located at the β-subunit position consisted of glycoprotein, which is a β-subunit.

Next we examined the effect of papain on two other (Na,K)-ATPases from different sources: one from *N. japonica*, the other from *A. salina*. These enzymes were incubated at 25°C for 6 h in the presence of different concentrations of papain. The residual activities and the electrophoretograms of the resulting enzymes are shown in table 1 and fig.5, respectively. In both cases, the bands corresponding to the β-subunit were recognized even when 10 μg/ml papain was added where the remaining activities were very low.

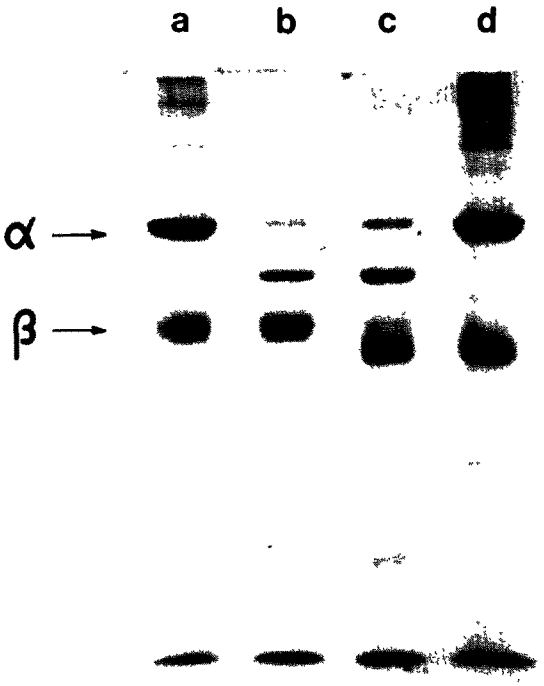

Fig.4. Sialidase treatment of papain-digested (Na,K)-ATPase. (Na,K)-ATPase (1 mg/ml) digested with 10 μg/ml papain at 25°C for 6 h was centrifuged at 100000 × g for 15 min. The pellet was resuspended in 0.1 M citrate buffer (pH 5.5) and treated with sialidase at 37°C for 2 h. (a) Control, (b) papain digested but sialidase untreated, (c) papain digested and sialidase treated, (d) papain undigested but sialidase treated. Residual activities were 5, 10 and 80% of the control for b, c and d, respectively.

Table 1

(Na,K)-ATPase activity (%)

Papain (μg/ml)	*Narke japonica*	*Artemia salina*
0	100	100
1	73	89
10	2	22

For details, see legend to fig.5

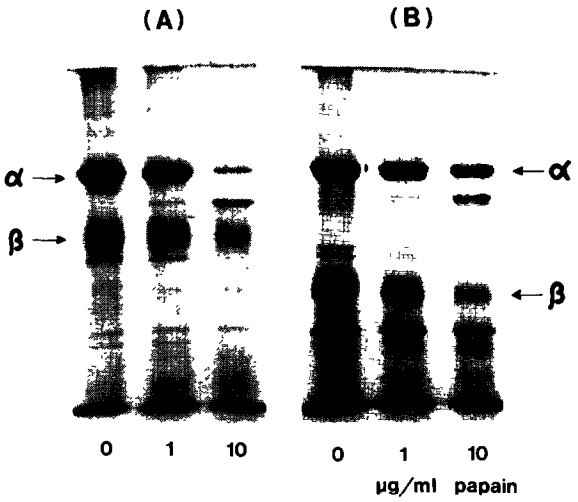

Fig.5. Papain digestion of *Narke* and *Artemia* (Na,K)-ATPase. (Na,K)-ATPases from *N. japonica* (A) and *A. salina* (B), both at 1 mg/ml, were digested with 1 or 10 μg/ml papain at 25°C for 6 h. The residual activity of each sample is shown in table 1.

4. DISCUSSION

There is much evidence suggesting that the β-subunit is an integral component of (Na,K)-ATPase although the functions of the subunit are still unknown. Our previous results that the reduction of disulfide bond(s) localized in the β-subunit results in the loss of enzymic activity [13] indicate that the subunit is essential for the expression of (Na,K)-ATPase.

As far as the enzymes from dog kidney and *N. japonica* are concerned, papain preferentially cleaves the α- rather than the β-subunit of (Na,K)-ATPases, leading to a progressive loss of activity. Although for the enzyme from *A. salina* the β-subunit seems to be digested in a more selective manner than is the α-subunit, as has been observed for the enzyme from avian nasal salt gland [15], it is unlikely that the β-subunit is selectively removed from the membrane.

Papain is still active even in the presence of SDS, judging from the result shown in fig.2. We tried to confirm the result using an artificial substrate for papain, *N*-benzyl-L-arginine-*p*-nitroanilide [24], but it failed due to the insolubility of the substrate in SDS.

Thermal denaturation of papain does not proceed instantaneously, and a few minutes are required for papain to be completely inactivated. Digestion of denatured subunits probably proceeds during the delay, and hence it is necessary to add acid such as perchloric acid to the digestion mixture for termination of the papain reaction.

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