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EVIDENCE FOR ESSENTIAL DISULFIDE BONDS IN THE β-SUBUNIT OF $(Na^+ + K^+)$-ATPase

MASARU KAWAMURA and KEI NAGANO

Department of Biology, Faculty of Science, Chiba University, Chiba and Department of Biology, Jichi Medical School, Yakushiji, Tochigi-ken (Japan)

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$(Na^+ + K^+)$-ATPase from dog kidney lost its activity when heated at 55°C in the presence of 0.3 M 2-mercaptoethanol. Either heat treatment alone or addition of reducing agent at around 25°C caused little inactivation. One disulfide bond per protomer (mol. wt. 146000) was reduced in the inactivated sample but in active samples no reduction occurred. Neither K^+-dependent phosphatase activity nor phosphoenzyme formation in the presence of Na^+ was detected in the inactivated sample, suggesting that the disulfide bond was essential for the catalytic cycle of $(Na^+ + K^+)$-ATPase. This essential disulfide bond belonged to the β-subunit, the glycoprotein component of the enzyme, indicating that the β-subunit may be an integral component of the $(Na^+ + K^+)$-ATPase system.

Introduction

It is well known that sulfhydryl groups (SH groups) of $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) have important roles in the catalytic cycle of the enzyme. Although the classification and effect of specific modifications of SH groups have been extensively investigated in relation to their possible role in the function of the enzyme (see references cited in Ref. 1), relatively little attention seems to have been given to the role of the disulfide bonds of this enzyme. The importance of the disulfide bonds has been demonstrated in the organization of several membrane proteins [2,3] and in the function of several hormone receptors [4–6]. Recently, the existence of two disulfide bonds important to the functioning of brush-border membrane D-glucose transporter has been reported [7]. These studies support the idea that disulfide bonds may play important roles in membrane protein functions.

In the present study we report that the disulfide bond in the glycoprotein component (β-subunit) of $(Na^+ + K^+)$-ATPase could be reduced only at high temperature. Reduction of the disulfide bond resulted in the inactivation of both $(Na^+ + K^+)$-ATPase and K^+-dependent phosphatase activity, indicating the important role of the disulfide bond in $(Na^+ + K^+)$-ATPase. Although the precise role of the subunit is far from being understood, the result reported here strongly suggests that the β-subunit is an integral component of the $(Na^+ + K^+)$-ATPase.

Materials and Methods

Enzyme preparation. Purified $(Na^+ + K^+)$-ATPase was obtained from dog kidney outer medulla by the method of Jørgensen [8] with a slight modification. The specific activity of the purified enzyme was in the range of 800–1200 μmol P_i/mg per h and the ouabain-insensitive ATPase was less than 1% of the total activity.

Labeling of the disulfide bonds. The number of disulfide bonds was estimated as the difference in

Abbreviations: SDS, sodium dodecyl sulfate; DACM, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide.

the amount of DACM which reacted with SH groups of the enzyme before and after treatment of the enzyme with 2-mercaptoethanol. Labeling of SH groups with DACM was performed in a medium containing 50 mM Tris-HCl buffer (pH 7.1), 1 mM EDTA, 1% SDS and 1–2 mg/ml protein at room temperature with 0.1 mM DACM [9]. After standing for 1 h samples were dialyzed against 50 mM Tris-HCl buffer (pH 9.0) containing 1 mM EDTA and 0.1% SDS for 2 days. The amount of reacted DACM was estimated from the molar absorbance at 380 nm (19800). The labeled enzyme was solubilized and electrophoresed in SDS and fluorescent bands on the gels were monitored by a chromatoscanner (Shimazu Scientific Instrument Co.) [9].

Other methods. $(Na^+ + K^+)$-ATPase and K^+-dependent phosphatase activities were measured as described elsewhere [10]. Protein determination was performed according to the method of Lowry et al. [11] using bovine serum albumin as the standard. Correction by quantitative amino acid analysis [12] was not made. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [13].

Materials. DACM, a fluorescent maleimide derivative, was purchased from Wako Pure Chemical Industries. DACM was dissolved in acetone and stored in a refrigerator. All other chemicals were of reagent grade or better quality.

Results and Discussion

Inactivation of $(Na^+ + K^+)$-ATPase with 2-mercaptoethanol at high temperatures

Purified enzyme in 50 mM imidazole buffer (pH 7.5) containing 3 mM ATP and 1 mM EDTA at a protein concentration of around 1.4 mg/ml was incubated for 15 min at various temperatures in the presence or absence of 0.3 M 2-mercaptoethanol. After the incubation, samples were diluted 10-fold with 25 mM imidazole buffer (pH 7.5) containing 1 mM EDTA and the residual activities were assayed. The result is shown in Fig. 1. Without 2-mercaptoethanol almost full activity remained unchanged up to 50°C. About 20% of the activity was lost at 55°C. In the concomitant presence of 2-mercaptoethanol, however, half of the activity was lost even at 50°C and no activity was left after treatment at 55°C. Fig. 2 shows the result of an experiment in which 2-mercaptoethanol concentration was varied. The concentration of 2-mercaptoethanol needed for complete inactivation at 55°C was rather high (0.3 M), but at the same concentration of the SH compound, little inactivation was observed in the enzyme treated at lower temperatures (25–30°C). The sensitization of the enzyme to 2-mercaptoethanol by heat may be due to a modification of the membrane structure so that 2-mercaptoethanol is accessible to the disulfide bond(s) of the enzyme. It may be possible that heat treatment around 50°C caused structural changes of the subunits of $(Na^+ + K^+)$-ATPase and/or of boundary lipid components surrounding the enzyme. Requirement of high concentrations of 2-mercaptoethanol for inactivation even under such a condition suggested that the disulfide bond(s) were buried in the occluded part of the enzyme. It has been shown that some disulfide bonds in the platelet integral membrane poly-

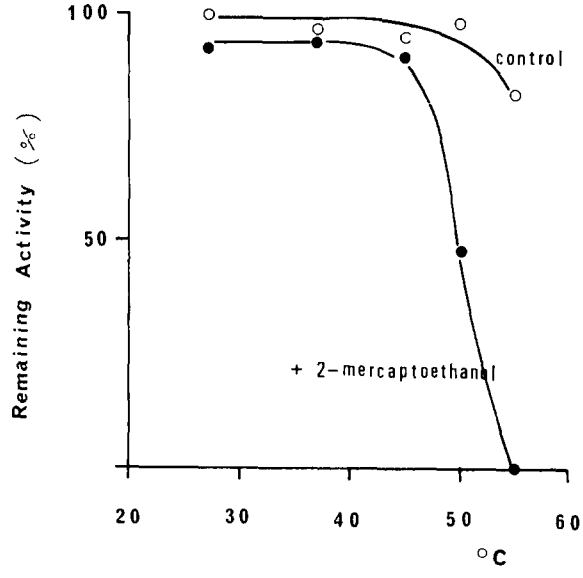

Fig. 1. Heat stability of $(Na^+ + K^+)$-ATPase. $(Na^+ + K^+)$-ATPase (1.5 mg/ml) was incubated at different temperatures in the medium containing 50 mM imidazole buffer (pH 7.5), 3 mM ATP and 1 mM EDTA in the presence (●) or absence (○) of 0.3 M 2-mercaptoethanol. After 15 min, samples were diluted 10-fold with chilled 5 mM imidazole buffer (pH 7.5)/1 mM EDTA and the residual activities were assayed. The sample volume used for the assay was 1/10 of the assay mixture, so that the concentration of 2-mercaptoethanol in the assay mixture was as low as 3 mM when present.

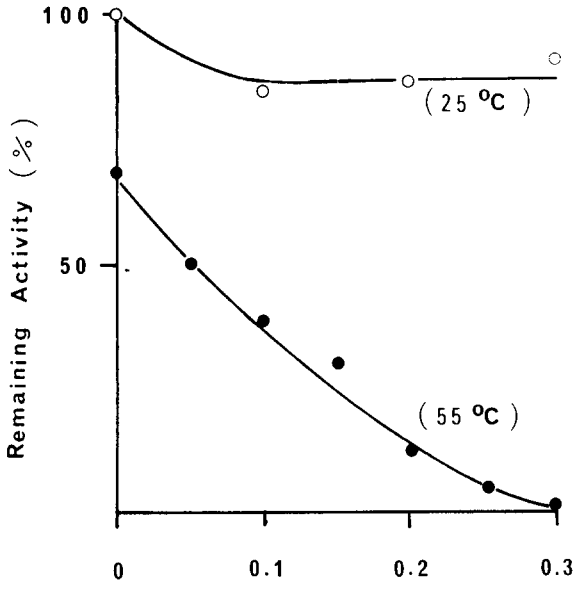

Fig. 2. Effect of the concentration of 2-mercaptoethanol on the $(Na^+ + K^+)$-ATPase activity. The conditions were the same as described in the legend for Fig. 1, except that the 2-mercaptoethanol concentration was varied. ○, 25°C; ●, 55°C.

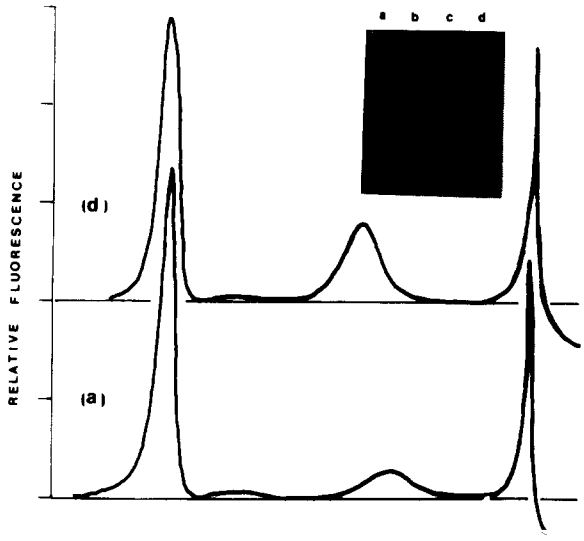

Fig. 3. SDS-gel electrophoresis of DACM-labeled $(Na^+ + K^+)$-ATPase. $(Na^+ + K^+)$-ATPase (1.3 mg/ml) was incubated for 20 min either at 30°C or at 55°C with or without 0.3 M 2-mercaptoethanol. Samples were then diluted with 1 mM EDTA and centrifuged at 38000 rpm for 30 min. The resulting pellet was dissolved in 1% SDS containing 50 mM Tris-HCl (pH 7.1), 1 mM EDTA and labeled with DACM as described in Materials and Methods. For each labeled sample, the same amount of protein was electrophoresed on a SDS slab gel of Laemmli's system. Two samples (a, d) out of four (a–d) shown in the inset were scanned with a chromatoscanner equipped with a fluorometer. a and b are the samples treated without 2-mercaptoethanol at 30°C and 55°C, respectively, and c and d are those treated with 2-mercaptoethanol at 30°C and 55°C, respectively.

peptide required a high concentration of reducing agent (5% dithiothreitol) to achieve complete reduction [3]. Dithiothreitol as well as 2-mercaptoethanol was effective in inactivation at 55°C, but ethanol had little effect, strongly suggesting that the inactivation (residual activity 81%) was due to the reduction of disulfide bond(s).

The inactivated enzyme showed neither K^+-dependent phosphatase activity nor phosphoenzyme formation from ATP in the presence of Na^+ (data not shown). All attempts so far to reactivate the inactivated enzyme have failed (data not shown).

Labeling of the disulfide bond(s)

To discover where the reduced disulfide bond(s) were located on the enzyme, we labeled SH groups of the enzyme treated at 30°C or 55°C in the absence or in the presence of 2-mercaptoethanol. As mentioned above, the activities were nearly completely retained, even after treatment, except in the case where the sample was treated at 55°C in the presence of 2-mercaptoethanol, when almost all activities were lost. The samples were then washed three times with 1 mM EDTA by centrifugation at 38000 rpm for 30 min to remove 2-mercaptoethanol. The samples were then labeled with DACM in the presence of 1% SDS. The presence of disulfide bond(s), if reduced, should be detected as the increment of the amount of SH groups labeled with DACM. The number of DACM molecules incorporated into a molecule (mol. wt. 146000) of the enzyme in the active state was 17.8 (average of 17.2 and 18.4 in two separate determinations) and 17.7 (18.2 and 17.2) for the samples treated at 55°C without 2-mercaptoethanol and at 30°C with 2-mercaptoethanol, respectively. The value was 20.0 (20.4 and 19.5) for the inactivated enzyme treated at 55°C in the presence of 2-mercaptoethanol. Since maleimide derivatives are reported to be nonspecifically incorporated into the phospholipid moiety of the enzyme [14], the numbers shown above did not necessarily represent the correct number of SH

groups in each sample. However, the difference in the number of DACM incorporated was near two between inactivated and active enzymes, suggesting that the reduction of one disulfide bond in an β-protomer of $(Na^+ + K^+)$-ATPase might cause its inactivation.

The samples were next analyzed by SDs-gel electrophoresis. For each labeled sample, the same amount of protein was loaded on a SDS-polyacrylamide slab gel. The result is shown in Fig. 3 where the fluorescence from the gel was monitored by a gel scanner equipped with a fluorometer. As shown in the inset in Fig. 3, no significant difference in the intensity of fluorescence was observed among four samples except that the intensity was increased for the β-subunit of the sample inactivated by heating in the presence of 2-mercaptoethanol. As is easily recognized on the figure, only the peak area of the β-subunit of the inactivated enzyme was increased. These observations indicated that the disulfide bond, whose reduction resulted in the inactivation of the enzyme, belonged to the β-subunit. No difference in intensity was observed between α-subunits treated with and without 2-mercaptoethanol, and the fluorescence from α-subunit was, therefore, due to free SH groups present in the native enzyme.

These results were in good agreement with the report by Esmann [1] who claimed that the number of disulfide bond in a molecule of $(Na^+ + K^+)$-ATPase from rectal glands of *Squalus acanthias* is only one and that the disulfide bond resides on the β-subunit. For the enzyme from dog kidney we previously reported the tentative number of 8 [15], which was recently re-estimated to be 1–2 [16]. The overestimation in our previous study had two main causes. First, upon the calculation of the number, the molecular weight of the enzyme was assumed to be 250 000, according to Askari et al. [17], which is larger than that adopted by Esmann (146 000). Recent molecular weight estimates for α- and β-subunits fall in the range 93 000–106 000 and 32 000–38 000 [18–20], respectively. The number should, therefore, be re-calculated. Second, it was recently found that in our enzyme preparation there was some substance other than $(Na^+ + K^+)$-ATPase subunits, perhaps oxidized phospholipid, which became reactive to *N*-ethylmaleimide or DACM after reduction with reducing agent in the presence of 1% SDS [16]. The substance was not completely released from the subunits even in the presence of 1% SDS. The alleged disulfide bond in the α-subunit that we reported [15] was due to this substance. In fact, no disulfide bond(s) seems to be present in the α-subunit of dog kidney $(Na^+ + K^+)$-ATPase according to the present data [16]. After these corrections, it is now concluded that only one to two disulfide bonds are present in a protomer of $(Na^+ + K^+)$-ATPase from dog kidney both being on the β-subunit.

The target of the 2-mercaptoethanol effect is, there, the β-subunit, which is clearly demonstrated in Fig. 3. The fact that reduction of the β-subunit resulted in the complete loss of all the activities tested indicates that the change of structure of the β-subunit could induce an accompanying structural change of the α-subunit, a catalytic subunit of $(Na^+ + K^+)$-ATPase, into its inactive state. This means that the β-subunit could affect the state of the α-subunit and that both subunits should be in close contact with each other. Although the precise function of the β-subunit is left obscure [21], the result in this paper strongly suggests that the β-subunit may be an integral component of the $(Na^+ + K^+)$-ATPase.

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