Biochimica et Biophysica Acta 821 (1985) 115–120
Elsevier

BBA 72793

Protective effect of Na^+ and K^+ against inactivation of $(Na^+ + K^+)$-ATPase by high concentrations of 2-mercaptoethanol at high temperatures

Masaru Kawamura[a,*], Keiko Ohmizo[a], Michiaki Morohashi[a] and Kei Nagano[b]

[a]*Department of Biology, Faculty of Science, Chiba University, Chiba 260 and* [b]*Department of Biology, Jichi Medical School, Yakushiji, Tochigi-ken 329-04 (Japan)*

(Received May 28th, 1985)

Key words: $(Na^+ + K^+)$-ATPase; Disulfide bond; Glycoprotein subunit; Mercaptoethanol

Purified dog kidney $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) was inactivated with high concentrations of 2-mercaptoethanol at 50–55°C. The inactivation was prevented by NaCl or KCl, with KCl being more effective than NaCl (the former ion being about one order more efficient under a typical set of experimental conditions). A disulfide bond in the β-subunit of the enzyme protein was prevented from reductive cleavage by NaCl or KCl in accordance with protection of the enzyme activity. Choline chloride did not exert a significant protective effect over a similar concentration range. $(Na^+ + K^+)$-ATPase was also inactivated with high concentrations of 2-mercaptoethanol in the presence of low concentrations of dodecyl sulfate. This inactivation was also prevented by NaCl or KCl, with the latter being again more efficient than the former. These results indicate that Na^+ and K^+ bound to their respective ion-binding sites on the α-subunit exert a protective effect on a disulfide bond on the β-subunit. This suggests some sort of interaction between the α- and the β-subunits.

Introduction

$(Na^+ + K^+)$-ATPase purified from canine kidney microsomes essentially consists of two kind of subunits, α and β; the relative molecular masses of the protein moieties being 121–94 kDa and 32–42 kDa, respectively [1–3]. The functional unit of the enzyme as a membrane ion transport device may be the dimer form of the heterodimeric protomer ($\alpha_2\beta_2$), although activity is detectable using the protomer form ($\alpha\beta$), at least in vitro [4–8]. Hitherto, all physiological features of the enzyme, such as location of binding sites for the transported ions (Na^+ and K^+), the energy-conferring substrate (ATP) and a specific inhibitor (ouabain), and the phosphorylation site in an intermediary reaction step, have all been attributed to the larger catalytic α-subunit [9]. The β-subunit is suggested to have an anchoring role when the nascent α-subunit is incorporated into the membrane [10–11]. No positive evidence indicating a definite significance in ATP hydrolysis or in ion transport function, however, has been unequivocally demonstrated for this smaller glycoprotein. In the case of avian nasal salt gland enzyme, based on an experimental result in which treatment of the membrane-bound $(Na^+ + K^+)$-ATPase by papain quickly makes the β-subunit undetectable while the hydrolytic activity of the enzyme remains intact [12], it was proposed that the β-subunit may be non-essential.

There are a few reports concerning disulfide

* To whom correspondence should be addressed at: Department of Biology, University of Occupational and Environmental Health, Kitakyushu 807, Japan.

Abbreviation: DACM, *N*-(7-dimethylamino-4-methylcoumarinyl)maleimide.

(S-S) bonds in the $(Na^+ + K^+)$-ATPase [13–15], although their actual roles in the enzyme are unclear. We have reported that a disulfide bond on the β-subunit is reduced by high concentrations of SH-agents such as dithiothreitol or 2-mercaptoethanol at high temperatures [15]. Concomitant inactivation of the enzyme, along with progressive reduction of the disulfide, is observed. Our proposal is that the β-subunit might play an essential role in the function of this enzyme. This interpretation is based on the fact that the disulfide bond is localized only on the β-subunit, whereas its reduction to thiols results in total inactivation of the enzyme.

We report in this paper than NaCl and KCl, but not choline chloride, prevented the enzyme from reduction of the disulfide with 2-mercaptoethanol, as well as concomitant inactivation, and that K^+ was more potent in this inhibition than Na^+. Several parts of the study have been reported elsewhere in preliminary form [16].

Materials and Methods

Enzyme preparations. Canine kidneys were generously supplied from the Animal Center of Jichi Medical School. ATPase was prepared from the microsomal fraction of kidney outer medulla by the method of Jørgensen [17] and assayed as described previously [13]. Final preparations had specific activities of 800–1200 μmol P_i released/mg protein per h. Ouabain-insensitive activity was usually less than 1% of the total ATPase activity.

Treatment of the enzyme with 2-mercaptoethanol at high temperatures. 1–2 mg/ml $(Na^+ + K^+)$-ATPase was incubated in 50 mM imidazole-HCl (pH 7.5) and 1 mM EDTA with or without 0.3–0.5 M 2-mercaptoethanol at the indicated temperatures, in a total of 100 μl. The incubation time was typically 10–15 min. The mixture was diluted 10-fold with cold 1 mM Tris-EDTA (pH 7.0), and an aliquot of 50 μl was added to the assay medium which contained 140 mM NaCl/14 mM KCl/5 mM $MgCl_2$/50 mM imidazole-HCl (pH7.5)/1 mM Tris-EDTA/3 mM Tris-ATP in a total of 500 μl. Thus, the original 2-mercaptoethanol was diluted 100-fold and its effect on $(Na^+ + K^+)$-ATPase activity was negligible in the final assay system.

Treatment of the enzyme with 2-mercaptoethanol in the presence of dodecyl sulfate. Tris dodecyl sulfate rather than sodium dodecyl sulfate (SDS) was used to avoid addition of unwanted Na^+. The incubation mixture was similar to that used for the high-temperature incubations, except for the additional presence of 0.2 mg/ml Tris dodecyl sulfate. The enzyme was treated in this mixture for 10–15 min at 25°C. Interruption of the incubation and assay of the enzyme activity were the same as in the high-temperature treatment.

Labeling and separation of $(Na^+ + K^+)$-ATPase subunit with N-(7-dimethylamino-4-methylcoumarinyl)maleimide. The enzyme preparation treated with 2-mercaptoethanol was thoroughly washed free of the SH-agent by repeated mixing with 1 mM Tris-EDTA (pH 7.0) and centrifugation. The enzyme was dissolved in 1% SDS/50 mM Tris-HCl (pH 7.1)/1 mM EDTA, and its SH groups were labeled with a fluorescent maleimide derivative, 0.1 mM *N*-(7-dimethylamino-4-methylcoumarinyl)maleimide (DACM) [18]. The labeled enzyme was dialyzed against 50 mM Tris-HCl (pH 9.0)/0.1% SDS/1 mM EDTA for 2 days, incubated in 2% SDS/50 mM Tris-HCl (pH 7.1)/1 mM EDTA overnight and separated through a Bio-Gel A column (1.5 m). The eluant buffer was 0.1 M Tris-HCl (pH 7.5)/0.1% SDS/1 mM EDTA. Fractions from the column were assayed for protein (by ultraviolet absorption at 280 nm) and DACM (by absorption at 380 nm) contents. The ratio A_{380}/A_{280} was taken as the index of specific incorporation of DACM into each subunit. Incorporation of DACM was also estimated from the intensity of fluorescence of the electrophoretically separated subunit bands on the polyacrylamide gel.

Protein was determined according to the method of Lowry et al. [19]. Polyacrylamide gel electrophoresis and other analytical methods for $(Na^+ + K^+)$-ATPase were performed as previously described [15].

Results

Inactivation of $(Na^+ + K^+)$-ATPase by 2-mercaptoethanol at high temperatures, and protection by Na^+ and K^+

$(Na^+ + K^+)$-ATPase is not an especially heat-

labile enzyme as long as it is treated in the particulate (membrane-embedded) form. The activity did not fall rapidly during incubation at 50–55°C. High concentrations of SH-agents such as 2-mercaptoethanol or dithiothreitol did not exert much inhibitory effect on the enzyme, provided that the treatment was performed at room temperature. When high temperatures and a high concentration of 2-mercaptoethanol (or dithiothreitol) were combined, however, rapid inactivation resulted [15]. Although the rate of inactivation was varied depending on the temperature, as well as on the concentration of the reducing SH-agent, and also, within a limited range, according to different enzyme preparations, under a typical set of conditions (50°C and 0.3 M 2-mercaptoethanol), complete inactivation was attained within 5 min (Fig. 1).

Addition of NaCl or KCl alleviated the inhibitory action of 2-mercaptoethanol treatment at the high temperature. When a sufficient concentration of Na^+ or K^+ was present, the inactivation was essentially prevented (Fig. 1). Half-maximal values of the prevention by cations ($K_{0.5}$ [Na^+] and $K_{0.5}$ [K^+]) were difficult to determine exactly, since the rate of inhibition was dependent on the incubation conditions as mentioned above, but in all cases K^+ was much more effective than Na^+. In a typical experiment (Fig. 2), $K_{0.5}$ [Na^+] and $K_{0.5}$ [K^+] were 13 and 1.3 mM, respectively. The protective effect seemed not to be a mere result of an increase in ionic strength, since the effect of choline chloride was weak even at 150 mM (Table I). Lithium ions exerted some degree of protection. ATP showed partial protection, but the specificity of the effect was questionable, since the degree of protection gradually increased for a wide range of ATP concentrations (0.1–10 mM); only 50% protection was attained at 10 mM, a level much higher than its saturation level as substrate for the enzyme.

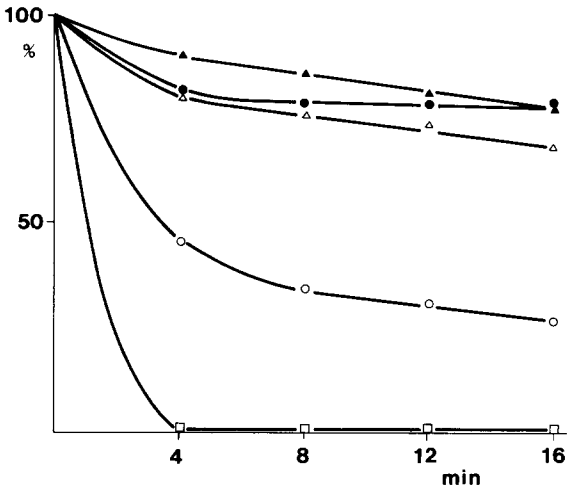

Fig. 1. Time-course of ($Na^+ + K^+$)-ATPase inhibition by 2-mercaptoethanol at 50°C and protection by NaCl and KCl. ($Na^+ + K^+$)-ATPase was treated with 0.3 M 2-mercaptoethanol at 50°C. Ordinate: remaining ($Na^+ + K^+$)-ATPase activity expressed in percent of control enzyme (treated without 2-mercaptoethanol) as 100%. □———□, 2-mercaptoethanol treatment without NaCl or KCl; ○———○, 15 mM NaCl; △———△, 15 mM KCl; ●———●, 150 mM NaCl; ▲———▲, 150 mM KCl.

Disulfide bonds in the α- and the β-subunits of 2-mercaptoethanol-treated enzyme

($Na^+ + K^+$)-ATPase treated with 2-mercaptoethanol was dissolved in 1% SDS/50 mM Tris-HCl (pH 7.1)/1 mM EDTA, and all available sulfhydryl groups in the enzyme were labeled with DACM. The labeled enzyme was separated into subunits through a Bio-Gel A column and the specific incorporation of DACM into each subunit was determined by its ultraviolet absorption (Fig. 3).

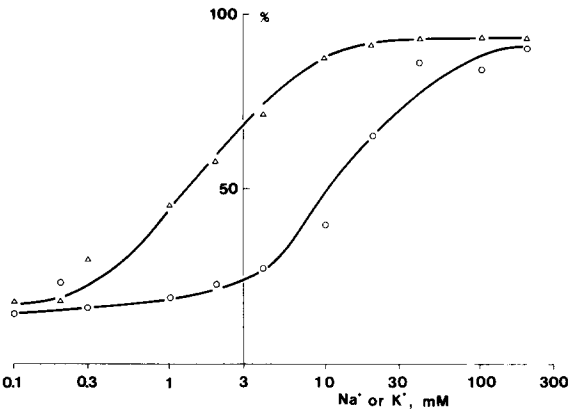

Fig. 2. Differential protective effect of NaCl and KCl against inactivation of ($Na^+ + K^+$)-ATPase by 2-mercaptoethanol treatment at 50°C. ($Na^+ + K^+$)-ATPase was treated with 0.3 M 2-mercaptoethanol at 50°C for 10 min in the presence of NaCl (○———○) or KCl (△———△). Remaining activity is expressed in percent of the control enzyme held at 20°C as 100%.

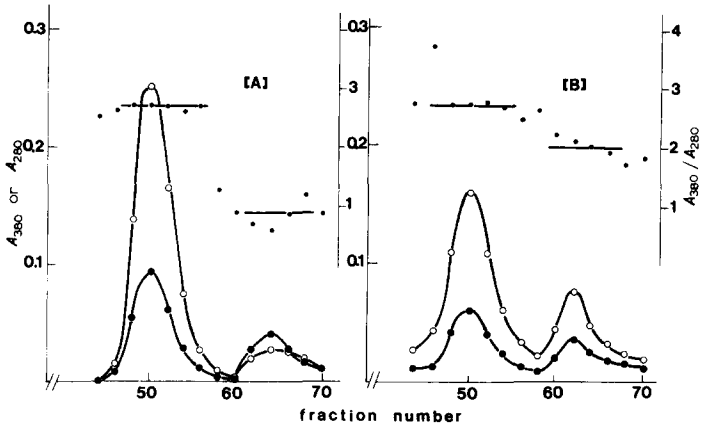

Fig. 3. Separation of $(Na^+ + K^+)$-ATPase α- and β-subunits labeled with DACM before and after 2-mercaptoethanol treatment. Enzyme was treated with 0.3 M 2-mercaptoethanol at 53°C and labeled with DACM. (A) Control enzyme before 2-mercaptoethanol treatment. (B) 2-mercaptoethanol-treated enzyme. Enzymes were solubilized in SDS and passed through a Bio-Gel A (1.5 m) column. ●———●, A_{280} (protein); ○———○, A_{380} (DACM); dots (.), ratio A_{380}/A_{280} (relative specific labeling of the subunit with DACM).

The ratio A_{380}/A_{280} was higher in the α-subunit, but the level was not changed by 2-mercaptoethanol treatment. In contrast, specific incorporation of DACM into the β-subunit was increased 2-fold by treatment with 2-mercaptoethanol.

Inactivation of $(Na^+ + K^+)$-ATPase at room temperature in the presence of dodecyl sulfate

We have already observed that $(Na^+ + K^+)$-ATPase rapidly lost its acitivity by treatment with 2-mercaptoethanol or dithiothreitol at room temperature if dilute SDS was present during the treatment [13]. Thus, it seemed significant to see whether Na^+ and K^+ would exert a similar protective effect here as was the case for the high-temperature treatment seen above. Tris dodecyl sulfate was as effective as SDS in inducing 2-mercaptoethanol-dependent inactivation of the $(Na^+ + K^+)$-ATPase. Na^+ and K^+ protected the enzyme against inactivation (Fig. 4). Exact $K_{0.5}$ values were difficult to obtain due to the same reason as described above concerning the high-temperature experiment, but it was evident that K^+ was again much more effective than Na^+.

Comparison of the levels of inactivation of $(Na^+ + K^+)$-ATPase activity with reduction of the disulfide bond

The above results suggested an inactivation mechanism where reduction of the disulfide bond on the β-subunit in the $(Na^+ + K^+)$-ATPase molecule would result in its activation. If this is true, the degree of inactivation would be in accordance with the increase of DACM on the β-subunit in partially inactivated enzyme preparations. To see whether this was indeed the case, various concentrations of K^+ were added to a series of incubation mixtures, and the subunits were separated by polyacrylamide gel electrophoresis. The level of DACM incorporated was estimated from the intensity of fluorescence from the gels under an ultraviolet lamp (Fig. 5). Comparing the labelings on the β-subunit in the absence (lane 3) and presence of a high concentration of KCl (40 mM, lane 7), suppression of the labeling on the β-subunit band is observed. Ouabain was essentially unable

TABLE I

EFFECT OF CATIONS AND ATP ON THE INHIBITION OF $(Na^+ + K^+)$-ATPase WITH 2-MERCAPTOETHANOL TREATMENT AT HIGH TEMPERATURES

Control enzyme held at 20°C without 2-mercaptoethanol treatment was taken as 100%. Treatment with 2-mercaptoethanol was for 10 min at 50°C in the presence of the cations or ATP as indicated.

Additions	(mM)	Remaining activity
None		6
NaCl	150	89
KCl	150	92
LiCl	150	42
Choline chloride	150	22
ATP	0.1	10
ATP	10	50

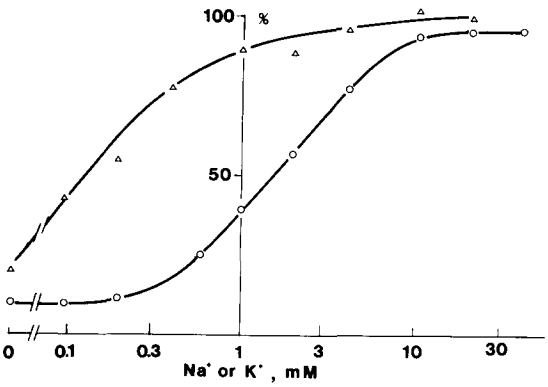

Fig. 4. Differential protective effect of NaCl and KCl against inactivation of $(Na^+ + K^+)$-ATPase by 2-mercaptoethanol treatment in the presence of Tris dodecyl sulfate. 1.4 mg/ml $(Na^+ + K^+)$-ATPase was treated with 2-mercaptoethanol at 25°C for 30 min in the presence of 0.2 mg/ml Tris dodecyl sulfate in 0.5 M 2-mercaptoethanol/50 mM imidazole buffer (pH 7.5), 1 mM Tris-EDTA and NaCl or KCl as indicated on the abscissa. Ordinate represents the remaining $(Na^+ + K^+)$-ATPase activity expressed in percent of control enzyme (treated without 2-mercaptoethanol) as 100. O——O, NaCl; △——△, KCl.

to protect the enzyme (Fig. 5), since fluorescence of the β-subunit treated with 2-mercaptoethanol in the presence of 1 mM ouabain was as intense as

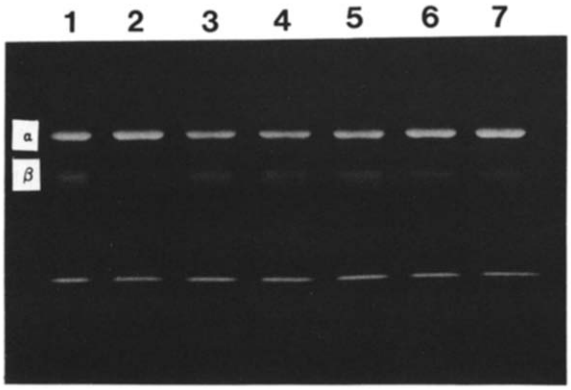

Fig. 5. Labeling of $(Na^+ + K^+)$-ATPase with DACM. 0.8 mg/ml $(Na^+ + K^+)$-ATPase was treated with 0.3 M 2-mercaptoethanol at 50°C for 10 min in the presence of various concentrations of KCl or 1 mM ouabain. After appropriate washing, the protein was labeled with DACM, and separated by electrophoresis on a slab gel plate (15 μg protein was applied in each well). The lanes are (from left to right): 1, ouabain added; 2, control (treated at 20°C); 3–7, treated in the presence of 0, 0.1, 1, 10 or 40 mM KCl. Control (lane 2) shows only a faint fluorescence on the β-subunit band. Ouabain (lane 1) does not interfere the DACM labeling.

that of the β-subunit treated in the absence of KCl.

Discussion

The number of disulfide bonds reduced in the present experiments was not directly determined. A 2-fold increase in DACM intensity due to newly exposed sulfhydryl groups on the subunit, however, is obvious (cf. Fig. 3). When the observation that two free sulfhydryl groups are on the β-subunit [14] is taken into consideration, the result indicates the presence of one reduced disulfide bond on the β-subunit.

Reduction of the disulfide bond on the β-subunit with concomitant loss of the catalytic activity may be taken as evidence, although still circumstantial, that the glycoprotein subunit is an essential component in the hydrolyzing function of the $(Na^+ + K^+)$-ATPase. What may be the substantial role of this smaller subunit in the reaction sequence is an interesting, but still an open, question. Our experiments using enzyme preparations from several sources (dog kidney, electric organ of the Japanese electric ray *Narke japonica*, and from the brine shrimp *Artemia salina*) show that the β-subunit was not selectively digested by papain and that no active enzyme preparations containing only the intact α-subunit are obtained [20].

The apparent necessity of an intact disulfide bond on the β-subunit for enzyme activity may be accepted as evidence for an α-β inter-subunit interaction in the catalytic function of $(Na^+ + K^+)$-ATPase, since all apparently functional sites reported up till now are located on the α-subunit. We suggest that the relatively higher efficiency of K^+ in comparison with Na^+ in the prevention of disulfide reduction correlates well with the lower $K_{0.5}$ for K^+ than that for Na^+ in the ATPase reaction. In this connection, a higher protective effect of K^+ compared to Na^+ has also been reported in the thermal denaturation of $(Na^+ + K^+)$-ATPase [21].

Prevention of thermal or detergent-dependent disulfide reduction by 2-mercaptoethanol with Na^+ and K^+, but not with choline chloride (cf. Figs. 2 and 4 Table I), may be taken as further evidence for an α-β interaction, since the specific ion-binding sites for Na^+ and K^+ are supposed, similar to

other functional sites, to be located on the α-subunit. The molecular basis of the interaction is, however, still unknown. Further investigation may reveal the nature of the interaction and the role of the apparently essential disulfide bond on the β-subunit of $(Na^+ + K^+)$-ATPase.

Acknowledgement

We thank Ms. A. Sugino of UOEH for typing the manuscript.

References

1 Cantley, L.C. (1981) Curr. Top. Bioenerg. 11, 201–237
2 Jørgensen, P.L. (1982) Biochim. Biophys. Acta 694, 27–68
3 Esmann, M. (1983) Curr. Top. Membrane Transp. 19, 67–81
4 Craig, W.S. (1982) Biochemistry 21, 5707–5717
5 Nakao, T., Fujitani, T. and Nakao, M. (1983) J. Biochem. 94, 689–697
6 Hayashi, Y., Takagi, T., Maezawa, S. and Matsui, H. (1983) Biochim. Biophys. Acta 748, 153–167
7 Brotherus, J.R., Jacobsen, L. and Jørgensen, P.L. (1983) Biochim. Biophys. Acta 731, 290–303
8 Esmann, M. (1984) Biochim. Biophys. Acta 787, 81–89
9 Post, R.L. (1983) Curr. Top. Membrane Transp. 19, 53–65
10 Sabatini, D., Colman, D., Sabban, E., Sherman, J., Morimoto, T., Kreibich, G. and Adesnik, M. (1981) Cold Spring Harbor Symp. Quant. Biol. 46, 807–818
11 Hiatt, A., McDonough, A. and Edelman, I.S. (1984) J. Biol. Chem. 259, 2629–2635
12 Freytag, J.W. (1983) FEBS Lett. 159, 280–284
13 Kawamura, M. Ohta, T. and Nagano, K. (1980) J. Biochem. 87, 1327–1333
14 Esmann, M. (1982) Biochim. Biophys. Acta 688, 251–259
15 Kawamura, M. and Nagano, K. (1984) Biochim. Biophys. Acta 774, 188–192
16 Kawamura, K. and Nagano, K. (1985) The Na Pump (Glynn, I.M. and Ellory, J.C., eds.), The Company of Biologists Ltd., Cambridge, in the press
17 Jørgensen, P.L. (1974) Methods Enzymol. 32, 277–290
18 Yamamoto, K., Sekine, T. and Kanaoka, Y. (1977) Anal. Biochem. 79, 83–94
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
20 Kamimura, K., Morohashi, M. and Kawamura, M. (1985) FEBS Lett. 187, 135–140
21 Fischer, T.H. (1983) Biochem. J. 211, 771–774