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STUDIES ON ($Na^+ + K^+$)-ACTIVATED ATPase

XLVI. EFFECT OF CATION-INDUCED CONFORMATIONAL CHANGES ON SULFHYDRYL GROUP MODIFICATION

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Summary

(1) ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.1.6.3) contains 34 sulfhydryl groups on the catalytic subunit, and two on the glycoprotein subunit. Under native conditions, only sulfhydryl groups on the catalytic subunit are accessible to modifying reagents.

(2) The degree of inhibition of ($Na^+ + K^+$)-ATPase activity by *N*-ethylmaleimide and 5,5'-dithiobis(2-nitrobenzoic acid) depends on the cations present in the reaction medium. Mg^{2+} strongly enhances the inhibitory effects of both sulfhydryl reagents. The effects of Mg^{2+} on the inhibition by 5,5'-dithiobis(2-nitrobenzoic acid) are counteracted by the addition of Na^+ or K^+. Na^+ has no more effect than choline on the inhibition by 5,5'-dithiobis(2-nitrobenzoic acid), but it enhances the inhibitory effect of *N*-ethylmaleimide at low Na^+ concentrations (less than 10 mM). Low concentrations of K^+ (less than 10 mM) slightly protect the enzyme against modification.

(3) Titration of residual sulfhydryl groups reveals that these ions do not only influence modification of essential sulfhydryl groups, but also that of sulfhydryl groups which are not essential for the enzyme activity.

(4) These results indicate that Na^+, K^+ and Mg^{2+} have marked effects on the

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Abbreviation: CDTA, *trans*-1,2-diaminocyclohexane tetraacetic acid.

conformation of the catalytic subunit of $(Na^+ + K^+)$-ATPase. Various enzyme conformations can be induced, depending on the concentration and the kind of cation added. The largest effects are observed after addition of Mg^{2+}.

Introduction

The enzyme, $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.1.6.3), which is involved in the active transport of Na^+ and K^+ across plasma membranes [1—4], contains essential sulfhydryl groups. Hence, treatment of the purified enzyme with sulfhydryl reagents leads to inhibition of its activity.

Important information about the mechanism of action of the enzyme has been obtained by comparing the rate of inactivation of the so-called partial reactions with that of the overall $(Na^+ + K^+)$-ATPase activity by sulfhydryl reagents [5—11]. Alternatively, the effects of specific ligands (ATP, Na^+, K^+, Mg^{2+}) on sulfhydryl group modified action by these reagents can be studied by measuring the rate of inactivation [5,7,8,12] and the number of modified sulfhydryl groups [13,14].

In this study we have combined the two approaches on a purified $(Na^+ + K^+)$-ATPase preparation from rabbit kidney outer medulla. We have previously characterized and classified the sulfhydryl groups essential for activity of this preparation [11]. Now we show that the essential sulfhydryl groups are located on the catalytic subunit. We also demonstrate that Na^+, K^+ and particularly Mg^{2+} induce conformational changes, depending on the nature as well as on the concentration of the ion. In addition, titration of the number of modified sulfhydryl groups indicates that the most reactive sulfhydryl groups are not essential for enzyme activity.

Materials and Methods

Enzyme preparation and assay

Purified $(Na^+ + K^+)$-ATPase is obtained from rabbit kidney outer medulla microsomes by extraction with sodium dodecyl sulfate and continuous sucrose density gradient centrifugation, as described by Jørgensen [15]. The preparation is freed from ATP and washed in 2 mM *trans*-1,2-diaminocylohexane tetraacetic acid (CDTA), 25 mM imidazole-HCl (pH 7.5) as described previously [11]. The specific $(Na^+ + K^+)$-ATPase activity of the preparations amounts to 1.5—2.0 mmol ATP hydrolysed/mg protein per h. Ouabain-insensitive activity is not detectable, and after sodium dodecyl sulfate gel electrophoresis only two bands (M_r 100 000 and 50 000) are observed.

$(Na^+ + K^+)$-ATPase assay has been carried out by determination of P_i released from ATP as described previously [11]. Protein determinations are performed according to the method of Lowry et al. [16] after trichloroacetic acid preparation as described by Jørgensen [15].

Chemical modification

Reactions with *N*-ethylmaleimide [10] and 5,5′-dithiobis(2-nitrobenzoic acid) [11] are carried out as previously described. The concentrations of these reagents and added ligands are indicated in the figures. Reagent concentrations

and incubation times are chosen in such a way that the effects of the ions on the degree of inactivation can readily be measured. Under standard conditions (Mg^{2+} + CDTA), the rate of inactivation follows pseudo-first-order rate kinetics down to a residual activity of 10%. The kinetics of inactivation have, however, not been examined at all combinations of ligands used in the present study. In all experiments the residual activity without added ligands is set at unity and the residual enzyme activity in the presence of a given concentration of the ligand is expressed as the ratio to residual activity in the absence of ligand.

Determination of the number of groups alkylated by *N*-ethylmaleimide is performed by incubation of (Na^+ + K^+)-ATPase with a mixture of *N*-[^{3}H]ethylmaleimide and *N*-ethylmaleimide of known concentration and specific activity. After incubation at 37°C, 30 μl samples of the *N*-ethylmaleimide reaction medium are diluted with 2 mM dithioerythritol, 25 mM imidazole-HCl (pH 7.5) at 0°C in order to stop modification. A 5 μl aliquot is used for (Na^+ + K^+)-ATPase-determination, whereas the remainder is precipitated by addition of 1 ml 10% trichloroacetic acid, containing 1 mM *N*-ethylmaleimide. The denaturated protein is collected by filtration over a Selectron AE 95 filter (1.2 μm pore size) and washed five times with 5 ml 5% (w/v) trichloroacetic acid. The filters are placed in 10 ml Aquasol (New England Nuclear, Boston, MA, U.S.A.) and ^{3}H is counted by liquid scintillation analysis. Blanks are prepared by addition of *N*-[^{3}H]ethylmaleimide after denaturation of protein by trichloroacetic acid.

Determination of the number of groups which have reacted with 5,5′-dithiobis(2-nitrobenzoic acid) is performed by measuring the absorbance of released 5-thio-2-nitrobenzoic acid at 412 nm as previously described [11]. The number of sulfhydryl groups per molecule of (Na^+ + K^+)-ATPase is calculated by assuming a molecular weight of 250 000 [17]. The true molecular weight appears to be somewhat higher (Peters, W.H.M., de Pont, J.J.H.H.M. and Bonting, S.L., unpublished data), which would make the number of sulfhydryl groups per molecule of enzyme proportionally higher.

Determination of tritiated protein

The distribution of the ^{3}H label over the two subunits of (Na^+ + K^+)-ATPase after treatment with *N*-[^{3}H]ethylmaleimide has been determined according to the method of Albanese and Goodman [18]. (Na^+ + K^+)-ATPase is treated with *N*-[^{3}H]ethylmaleimide under native conditions as well after solubilization in sodium dodecyl sulfate. The treated preparations are subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis in 8% polyacrylamide gels, as described by Laemmli [19]. After electrophoresis, the gel is cut in 1.5. mm slices. These are weighed in counting vials and dissolved in a 10:1 (v/v) mixture of H_2O_2 (concentrated) and NH_4OH (concentrated). When the slices are completely dissolved (usually after several days at room temperature), 10 ml of scintillation fluid (Aquasol) are added and the mixture is counted in a liquid scintillation analyser. Since the slices vary somewhat in thickness, the weight of the gel slices is used to calculate the relative mobility of the radioactive band. A parallel gel is stained with Coomassie blue in order to determine the position of the protein bands.

Materials

ATP and adenyl imidodiphosphate are obtained from Boehringer (Mannheim, F.R.G.), 5,5'-dithiobis(2-nitrobenzoic acid) from Sigma Chemical Co. (St. Louis, MO, U.S.A.), *N*-ethylmaleimide from Merck (Darmstadt, F.R.G.), and *N*-[^{3}H]ethylmaleimide (tritiated in the ethyl group, 100 Ci/mol) from the Radiochemical Centre (Amersham, U.K.). All other reagents are of reagent grade.

Results

Distribution of sulfhydryl groups over the subunits

The number of groups reacting with *N*-ethylmaleimide in each of the two subunits of ($Na^+ + K^+$)-ATPase depends on the reaction conditions. Under native conditions, *N*-[^{3}H]ethylmaleimide reacts only with groups located on the catalytic subunit (Fig. 1). When the enzyme is dissolved in 1% (w/v) sodium dodecyl sulfate prior to reaction, reactive sulfhydryl groups can also be demonstrated on the glycoprotein subunit. From the distribution of the tritium label over both subunits after reaction with detergent-solubilized enzyme, we calculate that 34 out of the total 36 sulfhydryl groups [11] are located on the catalytic (α) subunit, and only two on the glycoprotein (β) subunit.

Cation effects on inhibition by 5,5'-dithiobis(2-nitrobenzoic acid)

The extent of inhibition of ($Na^+ + K^+$)-ATPase activity by sulfhydryl reagents is influenced by the type of cations present as well as by their concen-

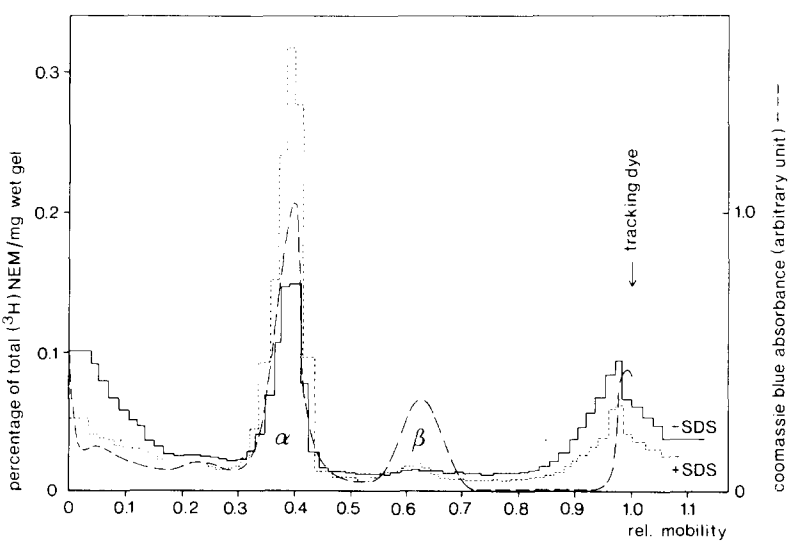

Fig. 1. Gel-electrophoretic pattern of ($Na^+ + K^+$)-ATPase labeled with *N*-[^{3}H]ethylmaleimide. ($Na^+ + K^+$)-ATPase is incubated for 30 min at 37°C in a medium containing 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA, 10 mM *N*-[^{3}H]ethylmaleimide (specific radioactivity 50 Ci/mmol), 1 mg protein/ml with (. . . . .) or without (———) 1% (w/v) sodium dodecyl sulfate (SDS). Excess *N*-[^{3}H]ethylmaleimide is then removed by gel filtration over a Sephadex G25 Coarse column (100 X 5 mm). When necessary, the protein is solubilized in 1% (w/v) sodium dodecyl sulfate. Polyacrylamide gel electrophoresis is performed according to the method of Laemmli [19]. The gels are sliced and the amount of ^{3}H in the slices is determined as described by Albanese and Goodman [18]. A control gel is stained with Coomassie blue (- - - - -).

tration. The inhibition by 5,5′-dithiobis(2-nitrobenzoic acid) depends on the ionic strength of the medium, addition of choline chloride or NaCl increasing the inhibition (Fig. 2a). However, the effects of KCl or $MgCl_2$ on the inhibition by 5,5′-dithiobis(2-nitrobenzoic acid) cannot simply be explained by an ionic-strength effect. Addition of up to 1 mM KCl results in a small but significant reduction in inhibition, whereas higher KCl concentrations increase the inhibition. Addition of $MgCl_2$ leads to an increased inhibition, which is significantly higher than that due to ionic-strength effects (Fig. 2b).

The effects of combined addition of cations are shown in Fig. 3. KCl and to a lesser extent NaCl antagonize the effect of $MgCl_2$. The effect of addition of K^+ reaches a maximal value at 5 mM, while Na^+ causes a gradually decreasing degree of inhibition over a 0—100 mM concentration range (Fig. 3a). Addition of Mg^{2+} in varying concentration in the presence of a constant 100 mM NaCl concentration results in increased inhibition (Fig. 3b), but the Mg^{2+} concentration reuired for half-maximal effect is higher than in the absence of NaCl (3 mM as against 1 mM; cf. Fig. 3b with Fig. 2b).

Cation effects on inhibition by N-ethylmaleimide

Different effects of the cations are observed for the inhibition of $(Na^+ + K^+)$-ATPase activity by *N*-ethylmaleimide. The increase in the ionic strength by addition of choline chloride or more than 50 mM NaCl has little effect on the extent of inhibition (Fig. 4a). Addition of low concentrations of NaCl increases the inhibition, maximally at 10 mM NaCl. Addition of up to 10 mM KCl decreases the inhibition, maximally at 5 mM (Fig. 4b). At higher concentrations KCl has no effect on the inhibition. Addition of $MgCl_2$ results in an increased inhibition, maximally at 10 mM $MgCl_2$.

Combined addition of cations to the *N*-ethylmaleimide reaction mixture has also been studied. Addition of Na^+ or K^+ in the presence of a constant (10 mM) $MgCl_2$ concentration tends to decrease the inhibition (Fig. 5a). These effects

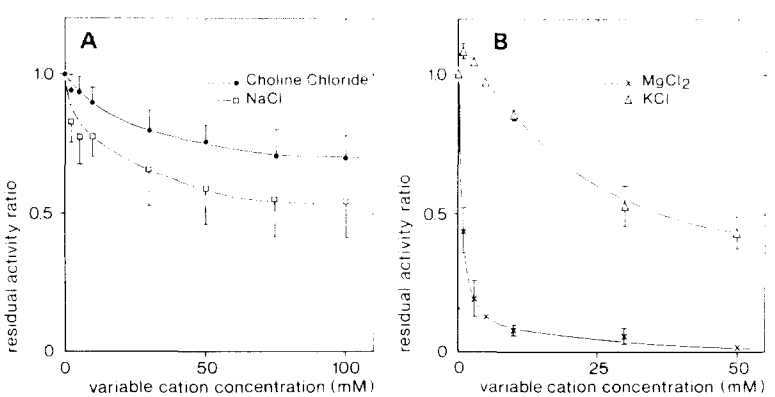

Fig. 2. Effects of various cations on inhibition of $(Na^+ + K^+)$-ATPase by 5,5′-dithiobis(2-nitrobenzoic acid). The reaction mixture contains 20 μM 5,5′-dithiobis(2-nitrobenzoic acid), 2 mM CDTA (omitted when $MgCl_2$ is added), 25 mM imidazole-HCl (pH 7.5), 0.5 mg protein/ml and the chloride salts in the stated concentrations. After incubation for 60 min at 37°C the mixture is diluted 20-fold with buffer, and is then assayed for $(Na^+ + K^+)$-ATPase activity as described in Materials and Methods. The ratio of the residual activities is plotted. At ratio 1.0 the residual activity is 65%. (A) Effets of choline chloride (●———●) and NaCl (□· — · — ·□). (B) Effects of KCl (△- - - - -△) and $MgCl_2$ (x———x).

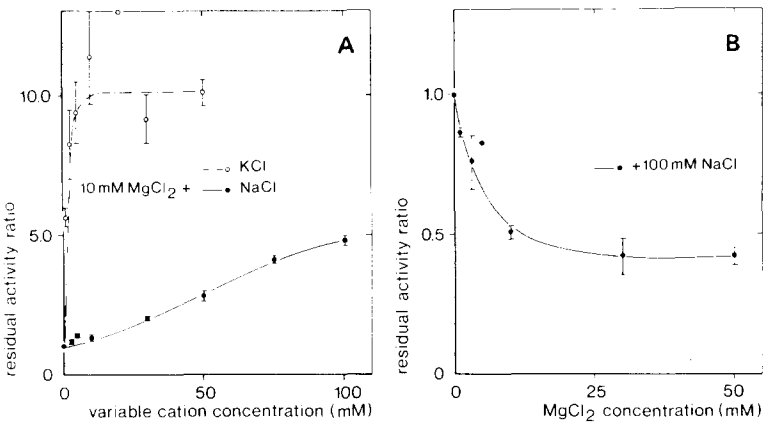

Fig. 3. Effects of cation combinations on inhibition of $(Na^+ + K^+)$-ATPase by 5,5'-dithiobis(2-nitrobenzoic acid). The reaction mixture contains 20 μM 5,5'-dithiobis(2-nitrobenzoic acid), 25 mM imidazole-HCl (pH 7.5), 0.5 mg protein/ml and the chloride salts in the stated concentrations. After incubation for 60 min at 37°C the mixture is diluted 20-fold with buffer, and is then assayed for $(Na^+ + K^+)$-ATPase activity as described in Materials and Methods. The ratio of the residual activities is plotted. (A) Effects of KCl (○· — · — ·○) and NaCl (●———●) in the presence of 10 mM $MgCl_2$. At ratio 1.0 the residual activity is 5%. (B) Effects of $MgCl_2$ (●———●) in the presence of 100 mM NaCl. At ratio 1.0 the residual activity is 45%.

are quite different from those of addition of Na^+ or K^+ alone (cf. Fig. 4a and b). Addition of increasing concentrations of $MgCl_2$ to the *N*-ethylmaleimide inhibition medium in the presence of a constant (100 mM) NaCl concentration increases the inhibition (Fig. 5b). However, the concentration of $MgCl_2$ required for maximal effect is higher than in the case of addition of Mg^{2+} alone (25 mM as against 10 mM; cf. Fig. 4b).

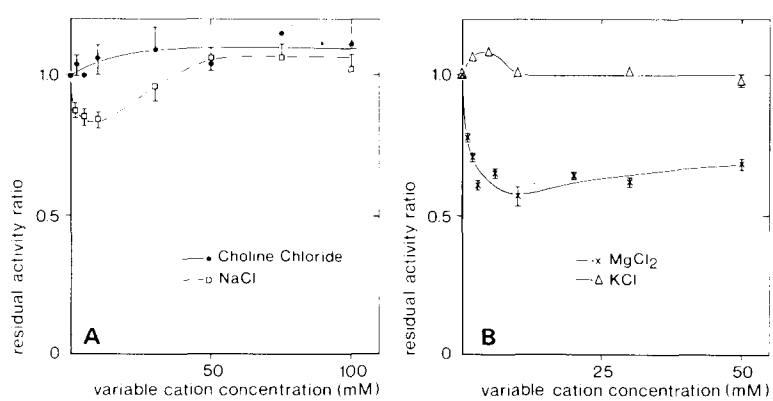

Fig. 4. Effects of various cations on inhibition of $(Na^+ + K^+)$-ATPase by *N*-ethylmaleimide. The reaction mixture contains 0.3 mM *N*-ethylmaleimide, 2 mM CDTA (omitted when $MgCl_2$ is added), 25 mM imidazole-HCl (pH 7.5), 7.5 μg protein/ml and the salts in the stated concentrations. After incubation for 30 min at 37°C, the reaction is stopped by addition of a 5-fold molar excess of dithioerythritol, and the residual $(Na^+ + K^+)$-ATPase activity is assayed as described in Materials and Methods. The ratio of the residual activities is plotted. At ratio 1.0 the residual activity is 65%. (A) Effects of choline chloride (●———●) and NaCl (□· — · — ·□). (B) Effects of KCl (△———△) and $MgCl_2$ (x———x).

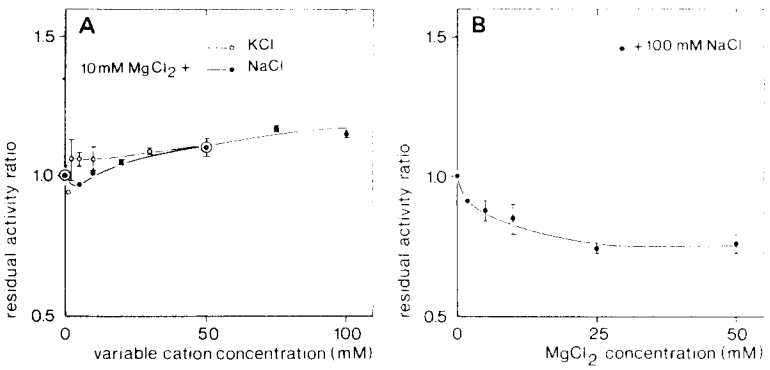

Fig. 5. Effects of cation combinations on inhibition of $(Na^+ + K^+)$-ATPase by *N*-ethylmaleimide. The reaction mixture contains 0.3 mM *N*-ethylmaleimide, 25 mM imidazole-HCl (pH.7.5), 7.5 μg protein/ml and chloride salts in the stated concentrations. After incubation for 30 min at 37°C, the reaction is terminated by addition of a 5-fold molar excess of dithioerythritol, and aliquots of the mixture are assayed for $(Na^+ + K^+)$-ATPase activity as described in Materials and Methods. The ratio of residual activities is plotted. At ratio 1.0 the residual activity is 70%. (A) Effects of KCl (○· — · — ·○) and NaCl (●————●) in the presence of 10 mM $MgCl_2$. (B) Effects of $MgCl_2$ (●————●) in the presence of 100 mM NaCl.

Cation effects on number of modified sulfhydryl groups

The relationship between the number of modified sulfhydryl groups and the residual $(Na^+ + K^+)$-ATPase activity has been studied for both sulfhydryl reagents. Modification with *N*-ethylmaleimide is carried out with various concentrations of the tritiated reagent and the residual enzyme activity is plotted as a function of the number of moles of *N*-ethylmaleimide bound per mole of $(Na^+ + K^+)$-ATPase. Fig. 6 shows an experiment in which either 100 mM NaCl or 10 mM $MgCl_2$ is present during modification; there is no marked difference between the two curves. Modification of the first four sulfhydryl groups does not lead to a large loss of enzyme activity, indicating that the most reactive

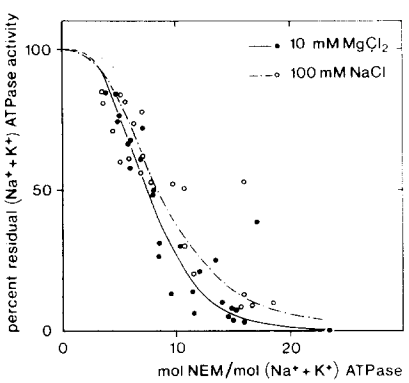

Fig. 6. Residual $(Na^+ + K^+)$-ATPase activity vs. number of groups modified by *N*-ethylmaleimide. The reaction mixture consists of 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA plus 100 mM NaCl (○· — · — ·○) or 10 mM $MgCl_2$ (●————●), 1 mg protein/ml and various concentrations of *N*-[^{3}H]ethylmaleimide (NEM) After incubation at 37°C for 30 min, the reaction is stopped by dilution of 30 μl incubation mixture with 1 ml 25 mM imidazole-HCl (pH 7.5), 2 mM dithioerythritol. Residual enzyme activity and moles of *N*-ethylmaleimide bound per mol $(Na^+ + K^+)$-ATPase are determined in aliquots of the reaction mixture as described in Materials and Methods.

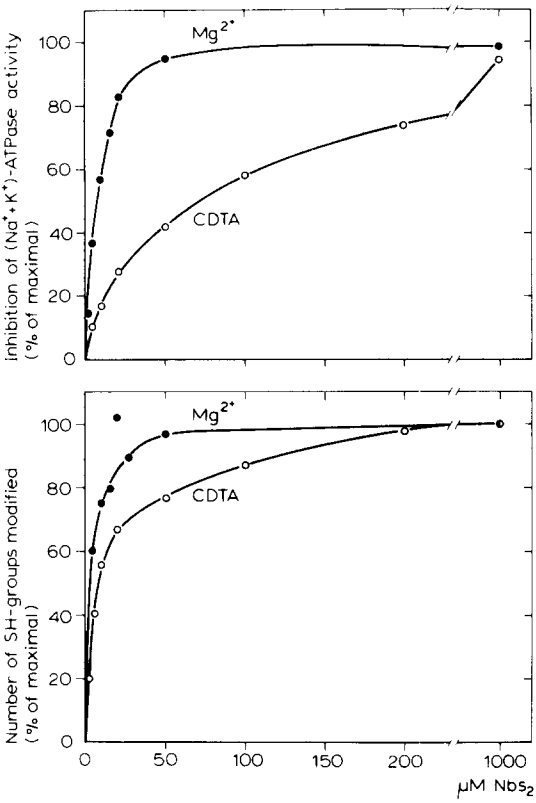

Fig. 7. Effect of Mg^{2+} and CDTA on the inhibition of $(Na^+ + K^+)$-ATPase and the number of sulfhydryl groups modified by 5,5′-dithiobis(2-nitrobenzoic acid). The reaction mixture consists of imidazole-HCl (pH 7.5), 2 mM CDTA (○———○) or 2 mM CDTA + 10 mM $MgCl_2$ (●———●), 0.2 mg protein/ml and 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) in the concentrations indicated. After incubation at 37°C for 1 h, a 50 μl aliquot is diluted three times with buffer and assayed for $(Na^+ + K^+)$-ATPase activity as described in Materials and Methods. The remaining incubation medium is centrifuged for 15 min at 16 000 × g and the number of sulfhydryl groups is determined from the absorbance at 412 nm. Curves represent averages from two experiments.

sulfhydryl groups are of minor importance for the enzyme activity. The maximum number of sulfhydryl groups which can be alkylated approaches the value of 26 previously determined by an indirect spectroscopic method [11]. The curves obtained for chemical modification in the presence of 2 mM CDTA, 5 mM $MgCl_2$ + 100 mM NaCl or 5 mM $MgCl_2$ + 100 mM NaCl + 6 mM adenyl imidodiphosphate do not differ significantly from those shown in Fig. 6. In order to obtain 50% inactivation, eight to ten sulfhydryl groups per enzyme molecule have to be modified.

Similar experiments have been carried out with 5,5′-dithiobis(2-nitrobenzoic acid), except that here the number of modified groups is determined spectroscopically. Fig. 7 shows that $(Na^+ + K^+)$-ATPase is inactivated at much lower reagent concentrations in the presence of Mg^{2+} than in the presence of CDTA. The number of modified sulfhydryl groups is also somewhat higher in the presence of Mg^{2+}. At 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) the enzyme is more than 95% inactivated both with and without Mg^{2+} present, and the num-

ber of modified sulfhydryl groups is also the same: with 10 mM $MgCl_2$ 11.0 mol/mol enzyme (S.E. 1.4, $n = 5$) and without Mg^{2+} 10.8 (S.E. 1.1, $n = 5$) mol/mol enzyme. The number of sulfhydryl groups to be modified in order to obtain 50% inhibition of $(Na^+ + K^+)$-ATPase is in both cases eight to ten per enzyme molecule. This again indicates that the extent of modification of a restricted number of essential sulfhydryl groups determines the activity of the enzyme.

Discussion

Sulfhydryl groups are essential for proper functioning of the $(Na^+ + K^+)$-ATPase reaction mechanism [5,8]. Previously, we have shown that $(Na^+ + K^+)$-ATPase contains 36 sulfhydryl groups per mol enzyme. Under native conditions only 12 of these react with 5,5′-dithiobis(2-nitrobenzoic acid), whereas 26 (including the 12 mentioned before) react with *N*-ethylmaleimide. In both classes of sulfhydryl group (the 12 residues reacting with both reagents and the 14 reacting only with *N*-ethylmaleimide) there is at least one essential sulfhydryl group.

The different effects of cations on the degree of inactivation by the two sulfhydryl agent confirm the fact that these reagents act differently. Increasing the ionic strength of the reaction medium has no effect on the degree of inhibition caused by *N*-ethylmaleimide, but it enhances the inhibition caused by 5,5′-dithiobis(2-nitrobenzoic acid). Na^+ does not significantly increase the inhibition caused by 5,5′-dithiobis(2-nitrobenzoic acid), whereas the inhibition by *N*-ethylmaleimide is increased by low (10 mM) but not by high (greater than 50 mM) Na^+ concentrations. Low K^+ concentrations (less than 10 mM) slightly protect the enzyme activity against the effects of both reagents. High K^+ concentrations (greater than 10 mM) have no effect in the inhibition by *N*-ethylmaleimide, but increase the inhibition by 5,5′-dithiobis(2-nitrobenzoic acid). Mg^{2+} increases the inhibition by both reagents, but the effect is largest for 5,5′-dithiobis(2-nitrobenzoic acid). The Mg^{2+}-induced increase in the inhibition by 5,5′-dithiobis(2-nitrobenzoic acid) can be anatagonized by K^+ and Na^+ (Fig. 3a).

The cation effects on the inhibition by the two sulfhydryl reagents can be most readily explained by assuming cation-induced conformational changes in the enzyme. These conformational changes would change the exposure of the essential sulfhydryl groups, resulting in an enhanced or a decreased rate of inactivation. The biphasic effects observed for K^+ with both reagents and for Na^+ with *N*-ethylmaleimide support the existence on the enzyme of multiple binding sites with different binding constants for these cations. The existence of several types of binding sites for K^+ and for Na^+ have been concluded both from kinetic experiments [20—22] and from direct binding studies [23—25].

Effects of Na^+ and K^+ on the enzyme conformation have also been observed in studies involving nucleotide binding [26], tryptic digestion [27], fluorescent probes [28], intrinsic fluorescence emission [29] and arginine modification [30]. In our experiments the effects of Na^+ and K^+ are only noticeable at certain ion concentrations and they are relatively small. Effects of the combined presence of Na^+ and K^+ have not been investigated in this study.

The most significant cation effects on the inhibition of $(Na^+ + K^+)$-ATPase by the two sulfhydryl reagents are obtained with Mg^{2+}. These effects differ from those of the monovalent cations, indicating that Mg^{2+} induces another conformational state than that induced by Na^+ and K^+. Such a conformational state has also been suggested by Kuriki et al. [31] on the basis of calorimetric studies.

Combined addition of $MgCl_2$ and either NaCl or KCl shows no additive effect. The Mg^{2+}-induced increase in inhibition by 5,5′-dithiobis(2-nitrobenzoic acid) is antagonized by K^+, which by itself in high concentration enhances inhibition. Hence, competition appears to occur between Mg^{2+} and K^+ or Na^+ for each of the cation-induced conformational states of the enzyme. Similar effects of these cations have been demonstrated on the dissociation rate constant of the enzyme-ouabain complex of rabbit kidney cortex [32]. Whether there is a real competition between these cations in inducing their own particular conformational state, or whether a new conformational state occurs, determined by the presence of two different cations, cannot be established from these experiments.

We have also correlated the effects of the sulfhydryl reagents on the enzyme activity with the number of sulfhydryl groups modified by them. For both reagents the most reactive sulfhydryl groups do not have much influence on the enzyme activity. Modification of four sulfhydryl groups per enzyme molecule leads to only 10% loss in enzyme activity, but modification of eight to ten sulfhydryl groups results in 50% inhibition.

Although the total number of reactive sulfhydryl groups is markedly different from the two sulfhydryl reagents [11], the number of sulfhydryl group modifications which leads to 50% inhibition is approximately the same in these two cases. From the different effects of cations on the extent of modification and from previous experiments in which the two reagents were given successively [11] it seems likely that different sulfhydryl groups are involved in the two inactivation processes.

When 5,5′-dithiobis(2-nitrobenzoic acid) is used in concentrations giving incomplete inhibition of $(Na^+ + K^+)$-ATPase, more sulfhydryl groups are modified and more inhibition is obtained in the presence of Mg^{2+} than in its absence. This indicates that in the Mg^{2+}-induced conformation the essential sulfhydryl groups are more exposed. However, plots of the number of sulfhydryl groups modified against the residual enzyme activity for several incubation conditions do not show significant differences. This indicates that ions affect the rate of inactivation through a change in exposure of essential sulfhydryl groups, but the extent of inhibition is independent of the ions present and is mainly determined by the number of essential sulfhydryl groups modified.

Under native conditions, sulfhydryl groups reacting with *N*-ethylmaleimide are only found on the catalytic or α-subunit. This means that the observed changes in reactivity of these sulfhydryl groups are due to conformational changes of this subunit. These changes are produced by mere addition of certain cations, which implies that they can involve only small changes in free energy.

The absence of reactive sulfhydryl groups on the glycoprotein (β) subunit in the native explains why reaction with bifunctional sulfhydryl reagents, such as

Cu^{2+}-phenanthroline [33—37], bismaleimidomethyl ether [37] and *p*-azophenyl-*N*, *N'*-dimaleimide [37], results primarily in the formation of an α_2 dimer. On the other hand, in the presence of digitonin or Triton X-100, Cu^{2+}-phenanthroline also appears to lead to the formation of an $\alpha\beta$ dimer [35,38], since a reactive group on the β-subunit has now been exposed (Fig. 1).

In summary, this study indicates that sulfhydryl reagents are a suitable tool for the detection of several ion-induced conformational changes in $(Na^+ + K^+)$-ATPase, which presumably play an important role in the functioning of this enzyme in sodium transport.

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