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SULPHYDRYL GROUPS OF $(Na^+ + K^+)$-ATPase FROM RECTAL GLANDS OF *SQUALUS ACANTHIAS*

DETECTION OF LIGAND-INDUCED CONFORMATIONAL CHANGES

MIKAEL ESMANN

Institute of Biophysics, University of Aarhus, 8000 Aarhus (Denmark)

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1. Modification of the Class II sulphydryl groups on the $(Na^+ + K^+)$-ATPase from rectal glands of *Squalus acanthias* with *N*-ethylmaleimide has been used to detect conformational changes in the protein. The rates of inactivation of the enzyme and the incorporation of *N*-ethylmaleimide depend on the ligands present in the incubation medium. With 150 mM K^+ the rate of inactivation is largest ($k_1 = 1.73$ $mM^{-1} \cdot min^{-1}$) and four SH groups per α-subunit are modified. The rate of inactivation in the presence of 150 mM Na^+ is smaller ($k_1 = 1.08$ $mM^{-1} \cdot min^{-1}$) but the incorporation of *N*-ethylmaleimide is the same as with K^+. 2. ATP in micromolar concentrations protects the Class II groups in the presence of Na^+ ($k_1 = 0.08$ $mM^{-1} \cdot min^{-1}$ at saturating ATP) and the incorporation is drastically reduced. ATP in millimolar concentrations protects the Class II groups partially in the presence of K^+ ($k_1 = 1.08$ $mM^{-1} \cdot min^{-1}$) and three SH groups are labelled per α subunit. 3. The K^+-dependent phosphatase is inhibited in parallel to the $(Na^+ + K^+)$-ATPase under all conditions, and the ligand-dependent incorporation of *N*-ethylmaleimide was on the α-subunit only. 4. It is shown that the difference between the Na^+ and K^+ conformations sensed with *N*-ethylmaleimide depends on the pH of the incubation medium. At pH 6 there is a very small difference between the rates of inactivation in the presence of Na^+ and K^+, but at higher pH the difference increases. It is also shown that the rate of inactivation has a minimum at pH 6.9, which suggests that the conformation of the enzyme changes with pH. 5. Modification of the Class III groups with *N*-ethylmaleimide—whereby the enzyme activity is reduced from about 16% to zero—shows that these groups are also sensitive to conformational changes. As with the Class II groups, ATP in micromolar concentrations protects in the presence of Na^+ relative to Na^+ or K^+ alone. ATP in millimolar concentrations with K^+ present increases the rate of inactivation relative to K^+ alone, in contrast to the effect on the Class II groups. 6. Modification of the Class II groups with a maleimide spin label shows a difference between Class II groups labelled in the presence of Na^+ (or K^+) and Class II groups labelled in the presence of K+ATP, in agreement with the difference in incorporation of *N*-ethylmaleimide. The spectra suggest that the SH group protected by ATP in the presence of K^+ is buried in the protein. 7. The results suggest that at least four different conformations of the $(Na^+ + K^+)$-ATPase can be sensed with *N*-ethylmaleimide: (i) a Na^+ form of the enzyme with ATP bound to a high-affinity site (E_1-Na-ATP); (ii) a Na^+ form without ATP bound (E_1-Na); (iii) a K^+ form without ATP bound (E_2-K); and (iv) an enzyme form with ATP bound to a low-affinity site in the presence of K^+, probably and E_1-K-ATP form.

Abbreviations: MSL, 3-(maleimidoethyl)-2,2,5,5-tetramethyl-1-pyrrolidinoxyl; α subunit, the 106kDa peptide; β subunit, the 40 kDa glycoprotein; γ subunit, the 10 kDa peptide; CDTA, *trans*-1,2-cyclohexylenedinitrilotetraacetic acid; MSH, 2-mercaptoethanol.

Introduction

In the preceding paper [1] it was shown that $(Na^+ + K^+)$-ATPase contains at least three classes of sulphydryl groups (SH groups) with different reactivities towards *N*-ethylmaleimide, and it was found that at least two classes could be distinguished from the biphasic time course for the inactivation by *N*-ethylmaleimide [1]. In the present paper the effects of Na^+, K^+ and ATP on the inactivation by *N*-ethylmaleimide are examined. The purpose is to use the observed different reactivities of the SH groups as a measure of the different conformations of the enzyme. ESR spectroscopy of a maleimide spin label (MSL) attached to the protein is used as an additional method for detecting conformational differences.

Methods

The methods used for most of the experiments have been described in the preceding paper [1].

Reaction with ^{14}C-labelled N-ethylmaleimide

^{14}C-labelled *N*-ethylmaleimide was obtained from Amersham International with a specific activity of about 12 Ci/mol as a pentane solution. It was stored at −20°C. The radioactive *N*-ethylmaleimide was diluted by addition of unlabelled *N*-ethylmaleimide in H_2O to the pentane solution of ^{14}C-labelled *N*-ethylmaleimide and the pentane was subsequently evaporated with N_2. Incubation at 37°C with enzyme and ligands was performed as described in [1] and after removal of an aliquot for measurement of enzyme activity the membranes were precipitated with 5% perchloric acid and washed twice in 0.1% trichloroacetic acid. The resulting pellet was hydrolyzed with 1 M NaOH for 30 min at 55°C or overnight at 37°C. About 25 μg were used for protein analysis (Lowry et al.) and about 50 μg for liquid scintillation counting. By this procedure the nonspecific binding of *N*-ethylmaleimide was reduced to less than 0.3 nmol/mg protein as determined either by addition of *N*-ethyl[^{14}C]maleimide which had been reacted with mercaptoethanol or by isotope dilution. In some experiments gel-filtration in Sepharose 6B in the presence of SDS was done in order to measure the *N*-ethylmaleimide incorporation into α, β and γ (see Ref. 1).

Results

Ligand effects on inactivation by N-ethylmaleimide

Class II. In the preceding paper [1] two classes of SH groups were established from the biphasic inactivation of $(Na^+ + K^+)$-ATPase incubated with *N*-ethylmaleimide in the presence of 150 mM K^+. The rate constants for the inactivation were 1.73 $mM^{-1} \cdot min^{-1}$ for Class II and 0.0107 $mM^{-1} \cdot min^{-1}$ for Class III. Fig. 1 shows that under conditions where practically only Class II reacts (0.1 mM *N*-ethylmaleimide) Na^+ will protect relative to K^+. It is also seen that ATP added in the presence of Na^+ leads to a much larger protection, as was expected from published experiments [2]. With K^+ + ATP in the incubation medium there is also protection relative to K^+ alone, but not to the same extent as with Na^+ + ATP. However, by incubation in the presence of K^+ + ATP there is an increased inactivation at longer incubation times (cf. Fig. 1), suggesting that the reactivity of the Class III groups in the presence of K^+ + ATP is larger than with K^+ alone (see below).

The inactivation by the reaction of the Class II groups follows pseudo-first-order kinetics when the enzyme is incubated with either 150 mM Na^+,

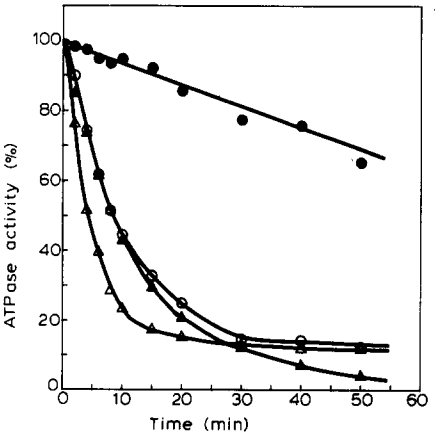

Fig. 1. Ligand-induced changes in reactivity towards *N*-ethylmaleimide. $(Na^+ + K^+)$-ATPase was treated with 0.1 mM *N*-ethylmaleimide in the presence of 150 mM K^+ (△——△) or Na^+ (○——○) with (▲, ●) or without 3 mM ATP (△, ○). Control enzyme without *N*-ethylmaleimide did not lose activity during the incubation times used. $(Na^+ + K^+)$-ATPase activity is expressed as percent of control enzyme activity, which was 1200 μmol/mg per h.

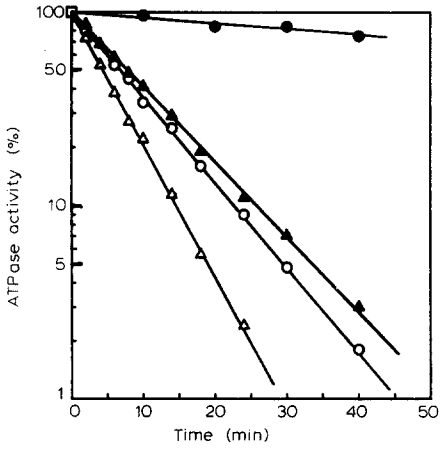

Fig. 2. Rapid-phase inactivation. The data from Fig. 1 are replotted after subtraction of the slow-phase inactivation in a semilogarithmic plot. The straight lines are extrapolated to $t=0$, giving an activity which is 92–95% of control activity. Symbols as in legend to Fig. 1.

150 mM K^+ or 150 mM K^+ + 3 mM ATP, Fig. 2. The points are obtained by subtracting the extrapolated slow phase from the initial rapid phase (see Ref. 1 for details). The inactivation seen with 150 mM Na^+ + 3 mM ATP is too slow with 0.1 mM *N*-ethylmaleimide to be analyzed kinetically (see below). The rate constants, k_1, obtained for each set of ligands are given in Table I, and it is seen that the phosphatase is inhibited in parallel with the ($Na^+ + K^+$)-ATPase.

TABLE I

REACTIVITY OF ATPase AND PHOSPHATASE TOWARDS *N*-ETHYLMALEIMIDE

The inactivation rate constants are given as first-order rate constants (in $mM^{-1} \cdot min^{-1}$) for the rapid phase (k_1 in the presence of 0.1 mM *N*-ethylmaleimide) and for the slow phase (k_2 with 5 mM *N*-ethylmaleimide). It is not experimentally possible to determine the rapid phase constants with 5 mM *N*-ethylmaleimide. S.E. is 5–8%, $n=4$.

	Rapid (k_1)		Slow (k_2)	
	ATPase	phosphatase	ATPase	phosphatase
Na^+	1.08	1.05	0.0092	0.0086
K^+	1.73	1.82	0.0107	0.0100
Na^+ + ATP	0.077	0.077	0.0024	0.0023
K^+ + ATP	1.00	1.02	0.021	0.0200

Class III. Under the conditions where the Class III groups react (e.g., 5 mM *N*-ethylmaleimide, see Fig. 4 in Ref. 1) the Class II groups will react so rapidly that (except for Na^+ + ATP) only the inactivation due to reaction with Class III groups can be followed with time. Fig. 3 shows that Na^+ protects relative to K^+, that Na^+ + ATP protects relative to Na^+, and that K^+ + ATP expose relative to K^+. The curves have been fitted by the same expression as given in the preceding paper for the inactivation in the presence of K^+ alone

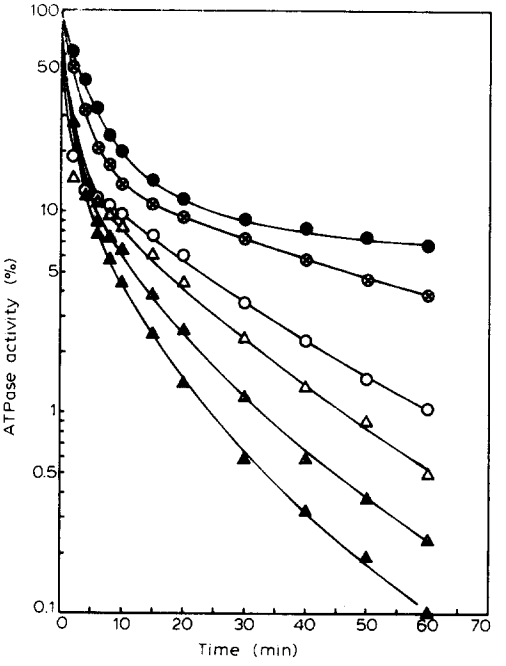

Fig. 3. Slow-phase inactivation. ($Na^+ + K^+$)-ATPase was treated with 5 mM *N*-ethylmaleimide in the presence of 150 mM K^+ (triangles) or Na^+ (circles) in the absence of ATP (○, △) or in the presence of 0.06 mM (⊗), 0.3 mM (⧋) or 3 mM ATP (●, ▲). Activity is expressed as percentage of control activity (1307 μmol/mg per h) in a semilogarithmic plot. The deviation from linearity at the longer incubation times is due to reaction of *N*-ethylmaleimide with the buffer used, which leads to a decrease in the *N*-ethylmaleimide concentration with time. The data are fitted by an expression of the form $A_t = A_0 \cdot \exp(-t \cdot k_2 \cdot N_0 \cdot \exp(-k_3 \cdot t))$ where A_0 is the enzyme activity when the Class II but not the Class III groups have been reacted (about 16%), A_t is the activity at a given time and the second-order rate constant k_2 is given in Table I for the ligand combinations used. N_0 (=5 mM) is the *N*-ethylmaleimide concentration at zero time. The term $N_0 \cdot \exp(-k_3 \cdot t)$ allows for the hydrolysis of *N*-ethylmaleimide and reaction with the histidine buffer used (see Ref. 1 for details).

(see legend to Fig. 3), indicating that a single class is reacted with *N*-ethylmaleimide when the activity is decreased from about 16% to zero. The rate constants, k_2, for the reaction of the Class III groups for each set of ligands are given in Table I, and it is seen that the $(Na^+ + K^+)$-ATPase and the phosphatase are inhibited in parallel. Fig. 3 also shows that the fraction of activity left when the Class II groups have reacted is the same (about 16%) even in the presence of 150 mM Na^+ + 3 mM ATP where the inactivation is the slowest. Figs. 1–3 show that there are two classes of SH groups necessary for activity, and that the ligands Na^+, K^+ and ATP can influence the rate constant for reaction with *N*-ethylmaleimide for both Class II and III. The fraction of activity left after reaction of the Class II groups is, however, the same no matter which ligand combination is used.

K^+ versus Na^+.

Figs. 1 and 2 show that the rate of inactivation with 0.1 mM *N*-ethylmaleimide is slower with Na^+ than with K^+. The Na^+/K^+ ratio giving half-maximal protection by Na^+ relative to K^+ was found by inactivating with *N*-ethylmaleimide for a fixed time (4 min) and substituting Na^+ for K^+, keeping the cation concentration constant at 150 mM (Fig. 4). The Na^+/K^+ ratio for 50% protection is 105:45, which is in agreement with the ratio obtained for half-maximal Na^+ activation of hydrolysis in the presence of 0.1 μM ATP [4], and thus probably reflects competition of Na^+ for K^+ on the inside site of the system where Na^+ activates (see Ref. 4).

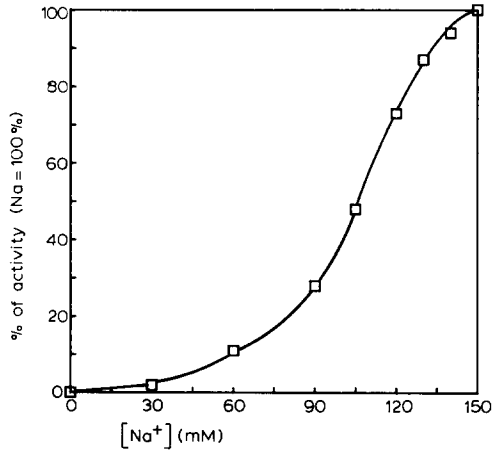

Fig. 4. Protection by Na^+ relative to K^+ towards inactivation with *N*-ethylmaleimide. Enzyme was incubated for 4 min with 0.1 mM *N*-ethylmaleimide with different concentrations of Na^+ and K^+, keeping the total cation concentration constant at 150 mM. The data are given as percentages, with the activity seen with 150 mM Na^+ as 100% and with 150 mM K^+ as 0%. The enzyme activity was subsequently measured at the optimal ligand combination (see Ref. 1). The Na/K ratio for 50% protection by Na^+ is 2.3 ± 0.3 (S.E., $n = 3$).

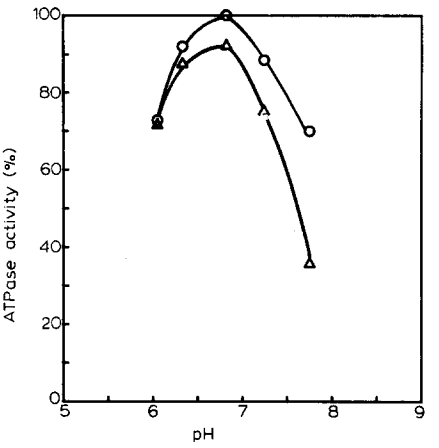

Fig. 5. Effect of pH on the inactivation with 0.1 mM *N*-ethylmaleimide. $(Na^+ + K^+)$-ATPase was incubated with 0.1 mM *N*-ethylmaleimide at different pH values with 150 mM Na^+ (○——○) or K^+ (△——△) present for 4 min at 37°C (see Methods). The reaction was stopped with MSH, and the $(Na^+ + K^+)$-ATPase activity was subsequently measured under standard conditions [1]. Control enzyme was stable during the time of incubation at all pH values. Activities are given as percentages of optimum with Na^+.

The different effects of Na^+ and K^+ on the rate of inactivation are pH-dependent (Fig. 5). At low pH (e.g., 6.1) there is practically no difference between the rates of inactivation, whereas the difference between Na^+ and K^+ is more marked at higher pH.

The reactivity of SH groups towards *N*-ethymaleimide increases with an increase in pH as the reacting group is deprotonated cysteine [5]. The inactivating effect of *N*-ethylmaleimide does not, however, increase monotonically with pH. Fig. 5 shows the effect of pH on inactivation with *N*-ethylmaleimide over a pH range from 6 to 8. The enzyme is protected at pH 6.8 relative to pH 6, both in the presence of Na^+ and K^+. An increase in pH above 6.8 leads to the expected

increase in inhibition, but the inhibition is more pronounced in the presence of K^+ than in the presence of Na^+.

Effects of ATP

Inactivation with 0.1 mM *N*-ethylmaleimide in the presence of 150 mM $Na^+ + 3$ mM ATP is about 15-times slower than with Na^+ alone (Table I). The slow reaction makes it difficult to analyse the shape of the curve, but with an increase in the *N*-ethylmaleimide concentration to 5 mM (see Fig. 3) it can be seen that the shape is biphasic, with a rapid and a slow phase similar to reaction with Na^+ alone.

ATP protects both in the rapid phase (Class II groups) and in the slow phase (Class III groups), the slow phase rate constant, k_2, being about 5-times smaller with $Na^+ + ATP$ than with Na^+ alone (Table I and Fig. 3).

Fig. 6 shows the protective effect of ATP at different concentrations with 0.1 mM *N*-ethylmaleimide. The ATP concentration necessary for half-maximal protection (K_{app}) is about 2 μM.

From Fig. 1 it is seen that there are two effects of ATP in the presence of 150 mM K^+. The rapid inactivation is slowed down, whereas the slow-phase inactivation is accelerated by ATP (Figs. 1 and 3 and Table I). The protective effect of ATP

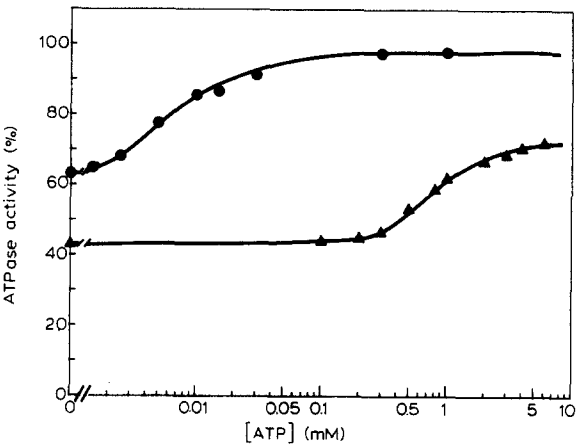

Fig. 6. Protection by ATP towards *N*-ethylmaleimide inactivation. $(Na^+ + K^+)$-ATPase was incubated for 5 min with 0.1 mM *N*-ethylmaleimide in the presence of 150 mM Na^+ (●——●) or K^+ (▲——▲) and varying ATP concentrations. The $(Na^+ + K^+)$-ATPase activity was subsequently measured, and is given in percentage of control.

on the rapid phase inactivation in the presence of 150 mM K^+ was investigated as with $Na^+ + ATP$ (see above) and the result is shown in Fig. 6. Low concentrations of ATP have little effect, and half-maximal protection is obtained with about 0.7 mM ATP, which is about 300-fold more than required in the presence of Na^+ (see Fig. 6). ATP

TABLE II

INCORPORATION OF *N*-ETHYL[^{14}C]MALEIMIDE INTO THE SUBUNITS OF THE $(NA^+ + K^+)$-ATPase

Enzyme activity is given in percent; control and incorporation as nmol/mg protein. E is native enzyme and E_{NEM} is enzyme prelabelled with non-radioactive *N*-ethylmaleimide (see text), Na^+ and K^+ are 150 mM, ATP is 3 mM and glycerol is 35% (w/v).

Enzyme	Ligands	[*N*-ethylmaleimide] (mM)	*t* (min)	temperature (°C)	Activity (%)	α	β	γ
E	$Na^+ + ATP$	0.1	1	37	95	13.5	8.8	28.7
E	$Na^+ + ATP$	0.1	3	37	90	17.9	11.7	34.7
E	$Na^+ + ATP$	0.1	20	37	75	23.1	15.2	36.7
E	Na^+	0.1	20	37	20	45.9	15.6	39.0
E	$Na^+ + ATP +$ glycerol	0.075	60	37	89	20.0	10.4	32.1
E	$Na^+ + ATP$	0.075	60	23	94	21.0	11.1	32.7
E	$Na^+ + ATP +$ glycerol	0.075	60	23	93	19.9	9.10	28.3
E_{NEM}	Na^+	0.1	60	37	17	42.4	3.1	6.6
E_{NEM}	K^+	0.1	60	37	18	32.2	2.8	9.7
E_{NEM}	$Na^+ + ATP$	0.1	60	37	63	15.9	3.1	6.8
E_{NEM}	$K^+ + ATP$	0.1	60	37	5	24.9	2.7	10.4

thus protects against the reaction of the Class II groups at a high-affinity site in the presence of Na^+, but at a low-affinity site in the presence of K^+. This is in agreement with the effects of Na^+ and K^+ on the affinity for the binding of ATP [6,7].

The inactivation due to reaction with Class III groups is influenced by ATP over the same concentration ranges as for the Class II group, that is, low concentrations (e.g., 0.02 mM) are sufficient for protection of Class III groups in the presence of Na^+, whereas much higher concentrations of ATP (e.g., 3 mM) are needed for the exposure of Class III groups in the presence of K^+ (Fig. 3).

N-Ethyl[^{14}C]maleimide incorporation

The effects of *N*-ethylmaleimide on the enzymatic activities were quantitated by the use of ^{14}C-labelled *N*-ethylmaleimide. Enzyme was reacted with 0.1 mM *N*-ethylmaleimide in the presence of Na^+ + ATP and of Na^+ alone (cf. legend to Fig. 1), and the incorporation of *N*-ethylmaleimide into the α, β and γ subunits (determined after gel filtration in SDS) is given in Table II. After 20 min the inactivation due to reaction with Class II groups in the presence of Na^+ is completed, and it is seen that about five SH groups per α are labelled, about one per β and about 0.4 per γ. Protection by 3 mM ATP in the presence of Na^+ reduces the labelling of the α subunit to about half, whereas the labelling of β and γ is the same. The inactivation of the (Na^+ + K^+)-ATPase by *N*-ethylmaleimide in the presence of Na^+ alone is thus related to the reaction of the SH groups on the α-subunit only.

However, gel filtration of enzyme reacted in the presence of the different ligands showed that the only partially pure enzyme contains some impurities capable of reacting with *N*-ethyl[^{14}C]-maleimide. This was to be expected from the titrations with *N*-ethylmaleimide and DTNB on the SDS-solubilized enzyme (see Ref. 1) as the number of SH groups in a $2\alpha + 2\beta$ unit was smaller than the number of SH groups per ^{32}P site in the membrane preparation.

Prelabelling

Reaction of enzyme with 75 μM *N*-ethyl[^{14}C]-maleimide in the presence of 150 mM Na^+/3 mM ATP/5 mM CDTA and with 35% glycerol present shows that at 23°C the enzyme can be reacted for 60 min with very little loss of activity (about 5%, Table II, rows 5–7). About two SH groups are labelled per α molecule, about one per β as well as some SH groups in proteins which are not part of the ATPase. These groups on α and β are called Class I groups [1]. In the following experiments with *N*-ethylmaleimide this prelabelling procedure followed by two or three centrifugations to remove ligands has been used to mask the Class I SH groups (with unlabelled *N*-ethylmaleimide) with almost no effect on the enzyme activity.

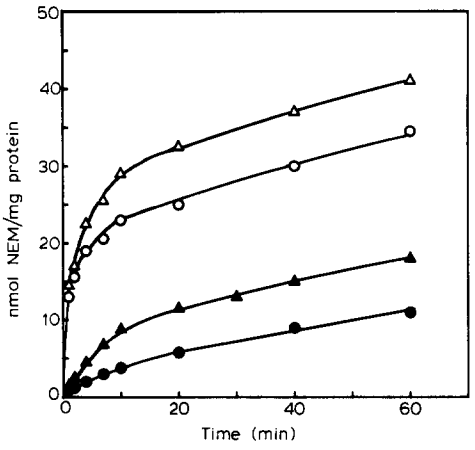

Fig. 7. The effect of prelabelling Class I SH groups. (Na^+ + K^+)-ATPase was reacted as given in Methods with 0.1 mM *N*-ethyl[^{14}C]maleimide in the presence of 3 mM ATP and 150 mM Na^+ (○——○) or K^+ (△——△). Filled symbols show the incorporation of *N*-ethyl[^{14}C]maleimide into enzyme which had been prelabelled with non-radioactive *N*-ethylmaleimide (NEM) in the presence of Na^+ + ATP + CDTA + glycerol (see text), and the ordinate gives the value in nmol/mg protein.

Fig. 7 shows the incorporation of *N*-ethylmaleimide under conditions where the activity is decreased (with K^+ + ATP, no glycerol) or protected (with Na^+ + ATP, no glycerol). The *N*-ethylmaleimide-incorporation into an enzyme which had been prelabelled with non-radioactive *N*-ethylmaleimide as above (and retained its activity) is also shown.

The difference between the incorporation in the presence of K^+ + ATP and Na^+ + ATP is about 6

nmol/mg, in agreement with the larger loss of activity with K^+ + ATP (cf. Fig. 1) and the difference is the same for the native and the prelabelled enzyme.

N-Ethyl[^{14}C]maleimide incorporated with different ligands

In order to measure the number of SH groups in Class II the prelabelled enzyme has been used to investigate the relationship between the inactivation seen with different ligands present (Fig. 1) and the corresponding incorporation of *N*-ethyl[^{14}C]maleimide (Fig. 8). The enzyme used is prelabelled as above and is inactivated by 0.1 mM *N*-ethyl[^{14}C]maleimide as a function of time and of the ligands present just as the native enzyme (see Fig. 1). As can be seen from Fig. 8, the incorporation of *N*-ethylmaleimide does not stop when the Class II groups have reacted. This is presumably due to the reaction of *N*-ethylmaleimide with groups other than Class II. A model

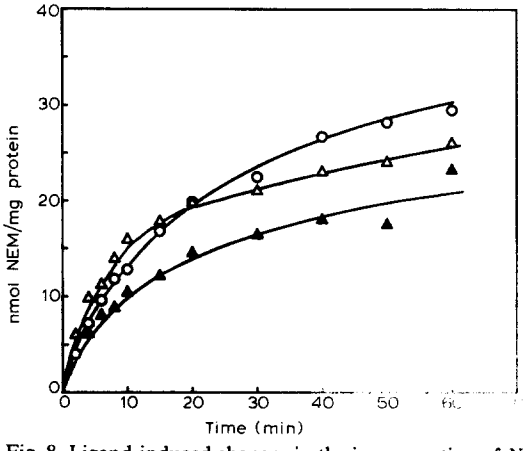

Fig. 8. Ligand-induced changes in the incorporation of *N*-ethylmaleimide (NEM) into (Na^+ + K^+)-ATPase. (Na^+ + K^+)-ATPase was reacted as in Methods with 0.1 mM *N*-ethylmaleimide in the presence of 150 mM K^+ (△——△), Na^+ (○——○) or K^+ + ATP (▲). The enzyme had been prelabelled with non-radioactive *N*-ethylmaleimide with little loss of activity; the specific (Na^+ + K^+)-ATPase activity after prelabelling was 1107 μmol/mg per h and the Na^+-dependent phosphorylation was 1.95 nmol phosphorylation/mg, which is taken as the enzyme concentration. Values are given as nmol *N*-ethylmaleimide/mg protein. The curves are of the form $Y = X_1 \cdot (1 - e^{-k_1 \cdot N_0 \cdot t}) + X_2 \cdot t$ (see text) and values for X_1 (in nmol/mg) and k_1 (in $min^{-1} \cdot mM^{-1}$) are: 15.6 and 1.6 for 150 mM K^+ (△——△), 15.6 and 1.1 for 150 mM Na^+ (○——○), and 11.8 and 1.1 for 150 mM K^+ + 3 mM ATP (▲——▲).

explaining the incorporation of *N*-ethylmaleimide from the data given in Fig. 8 must thus take the labelling of other SH groups than Class II into account. The data given in Fig. 8 can be fitted by the following simple expression:

$$Y = X_1 \cdot (1 - e^{-k_1 \cdot N_0 t}) + X_2 \cdot t$$

where Y is the number of SH groups reacted at given time, N_0 is the *N*-ethylmaleimide concentration, and X_1 is the total number of SH groups which can be reacted with the rate constant k_1, i.e., the Class II groups. The term $X_2 \cdot t$ reflects the time-dependent incorporation of *N*-ethylmaleimide into SH groups without importance for the enzyme activity, i.e., either SH groups on the impurities of the system or SH groups of Class I which were not labelled in the presence of 35% glycerol.

K^+. As expected from the activity measurements, enzyme incubated with K^+ alone shows the fastest incorporation of *N*-ethylmaleimide, and about 8 mol SH groups react ($X_1 = 8$) per mol enzyme, taking the number of phosphorylation sites as a measure of the enzyme concentration.

Na^+. With Na^+ alone in the medium, the incorporation in the rapid phase is a little slower than with K^+, in agreement with the slower loss of activity, and the number of SH groups reacted, $X_1 = 8$ mol/mol, is the same for Na^+ and K^+.

K^+ + ATP. In the presence of 150 mM K^+ + 3 mM ATP the rate of inactivation in the rapid phase is about the same as with 150 mM Na^+ alone (Figs. 1 and 2) but the incorporation of *N*-ethyl[^{14}C]maleimide is distinctly lower after 10–20 min of reaction, $X_1 = 6$ mol/mol, whereas $X_1 = 8$ for Na^+. This shows that high concentrations of ATP in the presence of K^+ leads to an enzyme form with two SH groups protected from reaction with *N*-ethylmaleimide, relative to Na^+ or K^+ alone. This is even more clearly seen at longer incubation times where enzyme incubated in the presence of K^+ + ATP has both the lowest activity (Fig. 1) and the smaller number of reacted groups (Fig. 8).

It is important to note that after 60 min of reaction more than 80% of the *N*-ethyl[^{14}C]-maleimide incorporated in the presence of Na^+ or K^+ labels the α-subunit (Table II rows 8–11). The

rest of the *N*-ethyl[^{14}C]maleimide is distributed equally on the proteins eluting between the α and γ subunits in the gel filtration and is independent of the ligands present. All changes in SH reactivity are thus confined to the α-subunit.

Spin labelling of Class II SH groups

Fig. 9 shows the ESR spectra of $(Na^+ + K^+)$-ATPase, prelabelled with *N*-ethylmaleimide as given above (i.e., masking the Class I groups) and subsequently reacted with 0.1 mM MSL in the presence of 150 mM Na^+ (A) or 150 mM K^+ + 3 mM ATP (B) for 20 min, thereby reacting all Class II groups and practically no Class III groups. The inactivation of the $(Na^+ + K^+)$-ATPase was about the same for both preparations. The MSL-labelled enzyme was then washed thoroughly by centrifugation (see Ref. 1) and resuspended in 30 mM histidine/150 mM NaCl/1 mM CDTA (pH 7.2) at 23°C. Spectrum B is clearly different from spectrum A, indicating that the SH groups reacting in the presence of K^+ + ATP are different from those reacting in the presence of Na^+ alone. The major difference between the two spectra is the smaller amount of strongly immobilized label (S) in spectrum B. The ratios of the line heights of strongly and weakly (W) immobilized labels (h_w/h_s) are 1.7 in the presence of Na^+, 3.33 with K^+ + ATP, and 1.45 with K^+ (spectrum shown in the preceding paper [1]). The spectra have not been quantitated, as the ESR instrument used is not equipped with an automated data collection facility. This precludes a further analysis of the differences between the spectra.

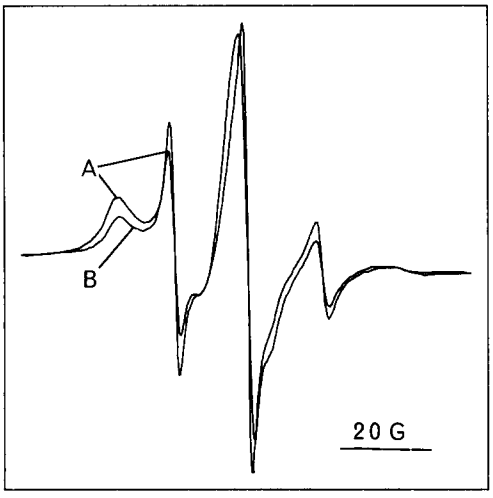

Fig. 9. ESR spectra of MSL-labelled $(Na^+ + K^+)$-ATPase. Enzyme which had been prelabelled with *N*-ethylmaleimide in the presence of Na^+ and glycerol with no loss of enzyme activity, was labelled with 0.1 mM MSL in the presence of 150 mM Na^+ (A) or 150 mM K^+ + 3 mM ATP (B) for 20 min at 37°C (see Ref. 1). The samples for ESR spectroscopy contained 10–14 mg protein/ml. The $(Na^+ + K^+)$-ATPase activity was 259 μmol/mg per h for A, and 228 μmol/mg per h for B.

It is, however, reasonable to assume that the incorporation of MSL in the presence of K^+ + ATP is lower than in the presence of K^+ alone, since the *N*-ethyl[^{14}C]maleimide labelling is lower with K^+ + ATP than with K^+ alone (see Fig. 7). This means that the higher h_w/h_s ratio for K^+ + ATP than for K^+ or Na^+ is related to a relatively lower incorporation of strongly immobilized label in the presence of K^+ + ATP than in the presence of K^+. Assuming that all the MSL is incorporated into the 6–8 Class II SH groups per $\alpha_2\beta_2$ (Table II) it is seen that some of the Class II SH groups must be located in an environment different from the rest of the Class II groups. This is unexpected, since the enzyme is inactivated in a monoexponential fashion when the Class II groups are reacted (see Ref. 1), showing that the Class II groups are kinetically identical.

Discussion

1. Titrations

More than 14 of the SH groups on $\alpha_2\beta_2$ are reactive towards *N*-ethylmaleimide in the membrane-bound form of the enzyme, in agreement with titrations with fluorescent probes [8,9]. About two groups on each α and one on β can be reacted with *N*-ethylmaleimide without altering the catalytic properties of the enzyme, Class I. In the presence of Na^+ or K^+ a further reaction of four SH groups on each α decreases the activity from 100% to about 16%. This is in agreement with titration data with fluorescent label [8] and *N*-ethylmaleimide on kidney enzyme [10], where it is shown that about 12 SH groups are reacted when the enzyme is inactivated. No attempt has been made to determine the extinction coefficient for the calibration of the method of Lowry et al. [11] with quantitative amino acid analysis. This could change the absolute values somewhat (see Refs. 12

and 13 for a discussion of this).

The $(Na^+ + K^+)$-ATPase and the K^+-dependent phosphatase activities are inhibited in parallel under all ligand combinations used, in agreement with the data reported by Schoot et al. [14], but in disagreement with the data of Wallick et al. [15]. The SH-group reactivity shows a cation-specific dependence of pH with an optimum protection at pH 6.8 at 37°C. This is in contrast to the behaviour of the kidney enzyme [16].

2. Effects of ATP

ATP in the presence of Na^+ protects against the incorporation of *N*-ethylmaleimide as well as the loss of activity (see also Ref. 2). The ATP concentration required suggests that ATP bound to a high affinity site (K_{app} about 2 μM) in the presence of Na^+ induces a conformation in which the essential groups—6–8 per $\alpha_2\beta_2$—are protected from reaction with *N*-ethylmaleimide. As ADP has the same effect as ATP (but with a 5-fold higher K_{app}) it is clear that the protected form of the enzyme is an E_1-ATP form and not a phosphorylated form. The large number of SH groups protected by ATP in the presence of Na^+ (about eight) suggests that the effect of ATP is to induce a certain conformation of the enzyme and not just a steric protection by ATP (see Ref. 2 for a discussion of this).

ATP can also protect the enzyme in the presence of K^+ but the affinity for ATP is about 300-fold lower and the protection is smaller. The protection by $K^+ + ATP$ occurs in the rapid phase of inactivation (Class II groups), whereas $K^+ + ATP$ expose the Class III SH groups on the enzyme relative to K^+ without ATP (Fig. 3). Enzyme incubated with *N*-ethylmaleimide in the presence of $K^+ + ATP$ retains about 60% of its ability to phosphorylate from ATP in the presence of $Na^+ + Mg^{2+}$, although the $(Na^+ + K^+)$-ATPase activity is reduced to less than 5% (Esmann and Klodos, unpublished data; see also Ref. 14). In the presence of $K^+ + ATP$ the incorporation of *N*-ethyl[^{14}C]maleimide is smaller than the incorporation with K^+ in the rapid phase (about one SH group per α). This means that Class II in the presence of $K^+ + ATP$ contains only six SH groups. The function of the two unlabelled SH groups is thus related to a retained ability of the enzyme to phosphorylate and is in agreement with the presence of SH groups at the ATP binding sites. (Patzelt-Wenczler et al. [17,18]).

3. Na^+-K^+-conformations

The different rates of inactivation with Na^+ and K^+ reflect different SH group reactivities of the Na^+ and K^+ forms of the enzyme. Enzyme in both forms is, however, inactivated to the same extent with 0.1 mM *N*-ethylmaleimide and with about the same number of SH groups involved, eight per $\alpha_2\beta_2$. An Na^+/K^+ ratio for 50% protection by Na^+ of 105:45 suggests that the effect is on the inside of the system, and is identical to the values previously found with *N*-ethylmaleimide [2].

The effect of ATP in millimolar concentrations in the presence of K^+ suggests that a K^+-ATP form of the enzyme is distinctly different from the Na^+ and K^+ forms, as evidenced by the dual effect on the rate of inactivation and the smaller number of SH groups reacting than in the presence of K^+ alone.

Spin labelling of enzyme in the presence of Na, K^+ or $K^+ + ATP$ shows that ATP in the presence of K^+ can protect one or two strongly immobilized SH groups from reacting with MSL. This suggests that the SH groups on the ATP binding site are buried in the protein. ESR spectroscopy also reveals that the Class II SH groups—although homogeneous by kinetic measurements—are inhomogeneous from the point of view of mobility of the SH groups.

4. A model

The above-mentioned data can be interpreted according to the scheme suggested by Karlish et al. [16].

$$E_1' \cdot Na \underset{ATP}{\rightleftharpoons} E_1 \cdot Na \rightleftharpoons E_1 \rightleftharpoons E_1K \underset{ATP}{\rightleftharpoons} E_2(K)$$

$E_1' \cdot Na$ is the enzyme form with ATP bound to a high affinity site in the presence of Na^+; $E_1 \cdot Na$ is the enzyme form seen in Na^+-rich media (without ATP); and $E_2(K)$ is the form seen in K^+-rich media (see Ref. 19). The cation concentrations used in the experiments are 150 mM, which is so much higher than the binding constants for Na^+ or K^+ (in the range of 1 mM, see Refs. 20–22) that there is virtually no E_1 (without

Na^+ or K^+ bound) present under the conditions used for modification with *N*-ethylmaleimide. The equilibrium between E_1K and $E_2(K)$ is poised far to the right in the absence of ATP, but can be displaced to the left by high concentrations of ATP (K_{app} 450 μM, see Ref. 19).

Our data suggest the following:

(1) Enzyme in the form $E_1' \cdot Na$ with ATP or ADP bound is protected from inactivation by *N*-ethylmaleimide and the rate constants for reaction with Class II and III groups are small, $k_1 = 0.077$ $mM^{-1} \cdot min^{-1}$ and $k_2 = 0.0024$ $mM^{-1} \cdot min^{-1}$, respectively. This conformation is induced by low concentrations of ATP or ADP (1–10 μM) in the presence of Na^+. A similar protected conformation can also be induced by glycerol or sucrose.

(2) Enzyme in the form $E_1 \cdot Na$ (150 mM Na^+ present) is inactivated much more rapidly ($k_1 =$ 1.08 $mM^{-1} \cdot min^{-1}$ and $k_2 = 0.0092$ $mM^{-1} \cdot min^{-1}$) than $E_1' \cdot Na$ (150 mM $Na^+ + 3$ mM ATP). As ATP protects eight SH groups per $\alpha_2\beta_2$ from reaction with *N*-ethylmaleimide this suggests that $E_1' \cdot Na$ is grossly different from $E_1 \cdot Na$.

(3) Enzyme in the form $E_2(K)$ (150 mM K^+ present) is inactivated more rapidly than $E_1 \cdot Na$: $k_1 = 1.73$ $mM^{-1} \cdot min^{-1}$ and $k_2 = 0.0107$ $mM^{-1} \cdot min^{-1}$. The ratio of the concentrations of Na^+ and K^+ which gives equal amounts of $E_1 \cdot Na$ and $E_2(K)$ (no ATP present) is about 105:45.

(4) Enzyme in the form $E_1 \cdot K$ has probably ATP bound to a low-affinity site (K_{app} 450 μM, see Ref. 19). At 3 mM ATP the equilibrium between E_1K and $E_2(K)$ is thus poised to the left. The inactivation by *N*-ethylmaleimide proceeds with $k_1 = 1.00$ $mM^{-1} \cdot min^{-1}$, which is about the same as for the $E_1 \cdot Na$ form, and $k_2 = 0.021$ $mM^{-1} \cdot min^{-1}$, which is larger than with Na^+ or any of the other ligand combinations used. The K_{app} obtained (0.7 mM) is in agreement with the value obtained from kinetic experiments [19]. $E_1 \cdot K$ is inactivated more rapidly in the slow phase than any of the other conformations, but fewer SH groups are blocked and the ability to phosphorylate from ATP is retained. This suggests:

(a) that ATP bound to the $E_1 \cdot K$ form protects only two SH groups from reaction with *N*-ethylmaleimide, whereas ATP bound to the E_1-Na form protects eight SH groups;

(b) that the two SH groups protected by $K^+ + ATP$ are essential for phosphorylation; and

(c) that the E_1K conformation of the enzyme is different from the other conformations of the enzyme in such a way that the SH groups in Class III are more reactive.

Tryptic modification of the enzyme has led to the conclusion that the form seen with Na^+ in the medium is the same as the form seen with $K^+ + ATP$ [23]. This seems also to be the case for the reactivity of the Class II groups as the rate constant k_1 is the same for $K^+ + ATP$ and Na^+, but this is not the case for the Class III groups (Table I). Another difference between the conformations sensed with trypsin and with *N*-ethylmaleimide is that the phosphatase activity is inhibited in parallel to the $(Na^+ + K^+)$-ATPase activity with *N*-ethylmaleimide, which is not the case with trypsin [24].

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References

1 Esmann, M. (1982) Biochim. Biophys. Acta 688, 251–259
2 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 234–245
3 Skou, J.C. (1979) Biochim. Biophys. Acta 567, 421–435
4 Skou, J.C. (1965) Physiol. Rev. 45, 596–617
5 Jocelyn, P.C. (1972) in 'Biochemistry of the SH-group', pp. 63–64 Academic Press
6 Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104–116
7 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5234–5240
8 Jesaitis, A.J. and Fortes, P.A.G. (1980) J. Biol. Chem. 255, 459–467
9 Gupte, S.S. and Lane, L.K. (1980) J. Biol. Chem. 255, 10362–10367
10 Schoot, B.M., Van Emst-De Vries, S.E., Van Haard, P.P.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1980) Biochim. Biophys. Acta 602, 144–154
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall,

R.J. (1951) J. Biol. Chem. 193, 265–275
12 Peters, W.H.M., Swarts, H.G.P., De Pont, J.J.H.H.M., Schuurmans Stekhoven, F.M.A.H. and Bonting, S.L. (1981) Nature 290, 338–339
13 Moczydlowski, E.G. and Fortes, P.A.G. (1981) J. Biol. Chem. 256, 2346–2356
14 Schoot, B.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1978) Biochim. Biophys. Acta 522, 602–613
15 Wallick, E.T., Anner, B.M., Ray, M.V. and Schwartz, A. (1978) J. Biol. Chem. 253, 8778–8786
16 Schoot, B.M., Schoots, A.F.M., De Pont, J.J.H.H.M., Schuurmans Stekhoven, F.M.A.H. and Bonting, S.L. (1977) Biochim. Biophys. Acta 483, 181–192
17 Patzelt-Wenczler, R. and Schoner, W. (1975) Biochim. Biophys. Acta 403, 538–543
18 Patzelt-Wenczler, R., Pauls, H., Erdmann, E. and Schoner, W. (1975) Eur. J. Biochem. 53, 301–311
19 Karlish, S.D.J., Yates, D.W. and Glynn, I.M. (1978) Biochim. Biophys. Acta 525, 230–264
20 Skou, J.C. and Esmann, M. (1980) Biochim. Biophys. Acta 601, 386–402
21 Karlish, S.J.D. (1980) J. Bioenerg. Biomembranes 12, 111–136
22 Hastings, D.F. and Skou, J.C. (1980) Biochim. Biophys. Acta 601, 380–385
23 Jørgensen, P.L.(1975) Biochim. Biophys. Acta 401, 399–415
24 Jørgensen, P.L. (1977) Biochim. Biophys. Acta 466, 97–108