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

TITRATIONS AND CLASSIFICATION

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1. $(Na^+ + K^+)$-ATPase from rectal glands of *Squalus acanthias* contains 34 SH groups per mol (M_r 265000). 15 are located on the α subunit (M_r 106000) and two on the β subunit (M_r 40000). The β subunit also contains one disulphide bridge. 2. The reaction of $(Na^+ + K^+)$-ATPase with *N*-ethylmaleimide shows the existence of at least three classes of SH groups. Class I contains two SH groups on each α subunit and one on each β subunit. Reaction of these groups with *N*-ethylmaleimide in the presence of 40% glycerol or sucrose does not alter the enzyme activity. Class II contains four SH groups on each α subunit, and the reaction of these groups with 0.1 mM *N*-ethylmaleimide in the presence of 150 mM K^+ leads to an enzyme species with about 16% activity. The remaining enzyme activity can be completely abolished by reaction with 5–10 mM *N*-ethylmaleimide, indicating a third class of SH groups (Class III). This pattern of inactivation is different from that of the kidney enzyme, where only one class of SH groups essential to activity is observed. 3. It is also shown that *N*-ethylmaleimide and DTNB inactivate by reacting with the same Class II SH groups. 4. Spin-labelling of the $(Na^+ + K^+)$-ATPase with a maleimide derivative shows that Class II groups are mostly buried in the membrane, whereas Class I groups are more exposed. It is also shown that spin label bound to the Class I groups can monitor the difference between the Na^+- and K^+-forms of the enzyme.

Introduction

The sulphydryl groups of the $(Na^+ + K^+)$-ATPase (EC 3.6.3.1) have been extensively studied, as they appear to play an important role in the catalytic cycle of the enzyme [1–19]. Modification has primarily been performed with maleimide derivatives [1–8,13,14,16–19] as well as DTNB [10,15] and Hg^+-containing fluorescence probes [12]. The presence of an SH group in the ATP-binding area of the α-chain has also been shown [9,10].

N-Ethylmaleimide modifies the $(Na^+ + K^+)$-ATPase in a manner which is dependent on the conformation of the enzyme [2–5,7,8] but the mechanism of inactivation appears to be complex [8,14]. *N*-Ethylmaleimide-modified enzyme exhibits an enhanced ADP-ATP exchange [3–5], and ATP has been shown to protect the enzyme against the inactivation by *N*-ethylmaleimide [3,8]. Modification with fluorescent maleimide-derivatives has been used to quantitate the reaction with the SH groups of the enzyme [16,18,19] and intramolecular distances between SH groups have been estimated with bifunctional maleimide derivatives [17].

Abbreviations: MSL, 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinoxyl; α subunit, the 106 kDa peptide; β subunit, the 40 kDa glycoprotein; γ subunit, the 10 kDa peptide; CDTA, *trans*-1,2-cyclohexylenedinitrilotetraacetic acid; SDS, sodium dodecyl sulfate; DTNB; bis(5-carboxy-5-nitrophenyl) disulphide; MSH, 2-mercaptoethanol.

The purpose of this and the following paper is to investigate the reaction with *N*-ethylmaleimide in greater detail and how the ligands Na^+, K^+ and ATP influence the reaction. The present paper reports titrations of SH groups and disulphide bridges of the α- and β-chains of the $(Na^+ + K^+)$-ATPase. It is shown that the inactivation of shark $(Na^+ + K^+)$-ATPase by *N*-ethylmaleimide cannot be described as a simple pseudo-first-order process. The data can be resolved into two exponential decays of activity, indicating at least two classes of SH groups of importance for the enzymic activity. A preliminary report of some of the results has appeared [20].

Methods

Preparation of membrane bound enzyme. $(Na^+ + K^+)$-ATPase was purified as previously described from rectal glands of the spiny dogfish (*Squalus acanthias*) (omitting saponin in the preparative procedure) to a specific activity of about 1400 μmol ATP hydrolyzed/mg protein per h [21]. ATPase and phosphatase activities were measured as before [21]. Control enzyme (without *N*-ethylmaleimide or DTNB added) was stable under the conditions used.

Protein determination. Protein was measured according to the method of Lowry et al. [22] using bovine serum albumin as standard. Spectroscopic measurements were carried out on a Varian-Cary 219. The 280 nm extinction coefficient of a 1 mg protein per ml solution (based on the procedure of Lowry et al. [22] with bovine serum albumin as a standard) was 0.83 for α and 1.11 for β.

SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out according to the methods of Weber and Osborn [23] using 7.5% gels. Staining was according to Fairbanks et al. [24]. The gels were scanned at 555 nm in a Beckmann Acta 3 with a scanning equipment and the aperture set at 0.1 mm.

N-Ethylmaleimide treatment. Membrane-bound enzyme was reacted with *N*-ethylmaleimide at 37°C in a buffer containing 0.8–1 mg/ml enzyme, 34 mM histidine (pH 7.4), 5 mM CDTA, 150 mM KCl and glycerol or sucrose as indicated in text. The reaction was initiated by addition of 1/10 vol. *N*-ethylmaleimide to the reaction mixture. Reaction was terminated by addition of 1 mM 2-mercaptoethanol, which stopped the reaction instantaneously. ATPase and phosphatase activities were subsequently measured.

Reaction with ^{14}C-labelled N-ethylmaleimide. The total number of the SH groups which could react with *N*-ethylmaleimide was measured as follows. Enzyme at a concentration of 1 mg/ml was solubilized in 2% SDS and 20 mM *N*-ethyl[^{14}C]maleimide was added at 37°C. After 5 and 10 h the reaction was stopped by addition of 2-mercaptoethanol and samples were gel-filtered in Sepharose 6B as described below. The amount of labelling was determined by radioactive counting and protein analysis (Lowry et al.) of the fractions eluted from the column. Radioactive *N*-ethylmaleimide was obtained from New England Nuclear.

DTNB treatment. Incubation at 37°C with DTNB was carried out as with *N*-ethylmaleimide (above). Reaction was terminated either by diluting 200-fold into the assay medium or by addition of 1 mM 2-mercaptoethanol. Addition of 1 mM 2-mercaptoethanol did not reverse the inactivation within 2 h.

Titration of SH groups in the presence of SDS was carried out as described in Ref. 25. The medium was 20 mM sodium phosphate (pH 7.5), 1 mM Na_2EDTA, 0.2% SDS and 0.6 mM DTNB. Membranes were solubilized in 2% SDS prior to titration and samples contained 0.2–0.3 mg protein/ml. The reaction was followed in a Beckmann Acta III spectrophotometer equipped with a constant-temperature cell at 23°C, and an extinction coefficient of 13100 $M^{-1} \cdot cm^{-1}$ was employed.

Gel-filtration. Gel-filtration was performed on a column (2.5 × 170 cm) of Sepharose 6B equilibrated with 20 mM sodium-phosphate (pH 7.5)/1 mM Na_2EDTA/0.2% SDS. Enzyme was solubilized in 2% SDS and heated to 100°C for 3 min prior to gel-filtration. The column was eluted at 10 ml/h and 5-ml fractions were collected.

Spin-labelling and ESR spectroscopy. $(Na^+ + K^+)$-ATPase was reacted with the spin-labelled maleimide derivative MSL, obtained from SYVA (3181 Porter Drive, Palo Alto, CA 94304, U.S.A.), under the same conditions as with *N*-ethylmaleimide (see above). The reaction was terminated by the addition of 0.1 mM MSH. The enzyme was

subsequently washed three times by centrifugation in 20 mM histidine/25% glycerol (pH 7.0) and the resulting pellet was resuspended at 10–14 mg/ml in 20 mM histidine buffer (pH 7.4) at 20°C. It was ascertained in two ways that all the MSL which had not reacted with protein was removed by the washing procedure: (a) the supernatant after the third centrifugation contained no free spin label as evidenced by ESR measurements, and (b) incubation with MSL which had been reacted with 2-mercaptoethanol before the incubation with the $(Na^+ + K^+)$-ATPase showed no detectable spin label in the last pellet, indicating a complete removal of non-covalently bound MSL.

Spectra were obtained on a Varian E-3 ESR spectrometer equipped with a variable temperature accessory. The spectrometer settings were: modulation amplitude, 1.0 G; modulation frequency, 100 kHz; microwave power, 12.5 mW; microwave frequency, 9.5 GHz; and temperature, 23 ± 1°C.

Results

Determinations of the SH content of the $(Na^+ + K^+)$-ATPase

The SH groups of the membrane bound enzyme were titrated with DTNB as described in Methods. It was observed that the reaction did not reach an endpoint after 24 h at 23°C, at which time about 50 nmol SH groups were titrated per mg protein. This prompted us to investigate the total number of SH groups and their localization on the subunits of the enzyme after denaturation and solubilization in SDS.

Titration with DTNB

$(Na^+ + K^+)$-ATPase, solubilized in 2% SDS, was titrated with DTNB as described in Methods, and an endpoint of reaction was reached within 6–8 h. The total number of SH groups on the enzyme was 99 nmol/mg protein, Table I. As the concentration of phosphorylation sites is 2.3 nmol/mg it can be calculated that there are 43 SH groups per enzyme molecule assuming one ^{32}P site per enzyme molecule. However, as the enzyme is only 60–70% pure it was necessary to isolate the subunits in order to obtain an exact figure of the total number of SH groups related to the $(Na^+ + K^+)$-ATPase.

The α and β subunits of the $(Na^+ + K^+)$-ATPase were isolated as described in Methods by gel filtration in Sepharose 6B in the presence of 0.2% SDS (Fig. 1). Peak I contains pure α-subunit as verified by SDS polyacrylamide gel electrophoresis, whereas peak II besides the β subunit contains some additional protein impurities. Only fractions containing more than 90% β subunit were used for titration with DTNB.

Fractions containing α or β were reacted with DTNB and an endpoint was reached within 6 to 8 h. When the titration was performed on the subunits from enzyme which was reduced with 2-mercaptoethanol or dithiothreitol in the presence of 2% SDS prior to gel filtration, 105 nmol SH groups were titrated per mg β subunit, whereas the number was 63 nmol without reduction (Table I). Using a molecular weight for the β-subunit of 40000 [26], this indicates the presence of two SH groups and one disulphide bridge in the β subunit. The number of SH groups in the α subunit was 141 nmol/mg both with and without reduction, corresponding to about 15 SH groups per molecule (M_r 106000; see Refs. 26, 27). This gives 34 mol of SH groups per mol $\alpha_2\beta_2$, which is in agreement with the SH content of the kidney enzyme [15]. This also shows that the impurities of the membrane-bound enzyme (about 30%) contain SH groups capable of reacting with DTNB of *N*-ethylmaleimide, namely the difference between

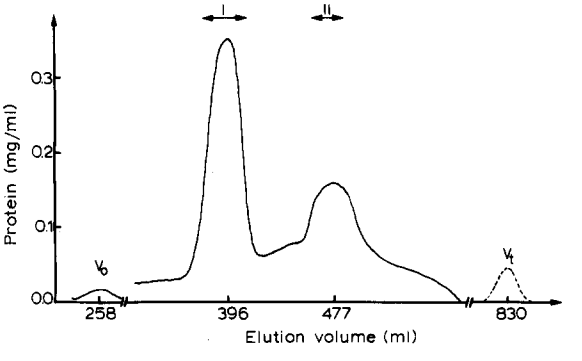


Fig. 1. Separation of the α and β subunits by gel-filtration in the presence of SDS. $(Na^+ + K^+)$-ATPase (16 mg protein) was treated as given in Methods, and the protein concentration is shown as a function of elution volume. SDS gels of the fractions show that area I consists of the 106 kDa peptide (α) whereas II consists of the 40 kDa peptide (β) plus some contaminating peptides (less than 10%).

TABLE I

INCORPORATION OF DTNB AND *N*-ETHYLMALEIMIDE INTO SDS-SOLUBILIZED ($Na^+ + K^+$)-ATPase AND THE DISTRIBUTION ON THE SUBUNITS

Values are given as nmol/mg (S.E., $n = 8$), and in brackets as mol/mol assuming M_r of the native enzyme to be 265000; M_r of α, 106000 and M_r of β, 40000. n.d., not determined.

	E	α	β	γ
DTNB	99.2±9.1 (43)	141±3.1 (15.0)	63±4.8 (2.5)	67±6.4
DTNB+2-mercaptoethanol	n.d.	131±4.2 (13.9)	105±7.5 (4.2)	83±8.3
N-Ethylmaleimide 5 h [a]	105 ±8.2 (46)	145±5.1 (15.4)	50±5.0 (2.0)	250±34
10 h [a]		143±8.2 (15.2)	48±3.2 (1.9)	400±46

(a) $n = 2$.

the 43 mol per mol phosphorylation site (see above) and the 34 mol per mol $\alpha_2\beta_2$.

A small peak of Lowry-staining material elutes after the β subunit in the Sepharose 6B column (the γ subunit, Ref. 28). It has not been shown that this protein definitely is a part of the ($Na^+ + K^+$)-ATPase, but it occurs in most purified ($Na^+ + K^+$)-ATPase preparations. On SDS-polyacrylamide gels this material runs as a band behind the tracking dye (M_r about 10000) and stains with Coomassie blue. Reaction with DTNB gives about 70 nmol SH groups per mg protein (Lowry) (Table I) for the γ subunits (0.8 mol/mol).

Titration with N-ethylmaleimide

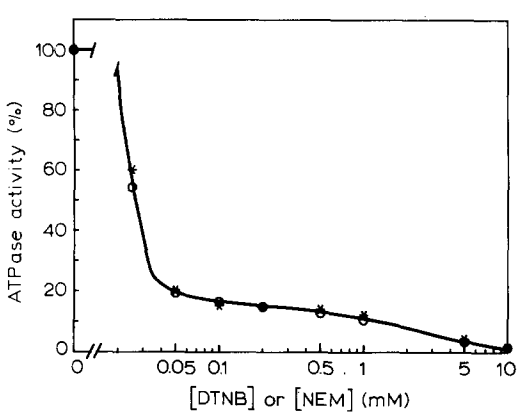
The total number of groups reactive towards *N*-ethylmaleimide in the presence of 2% SDS was determined by titration with radioactive *N*-ethylmaleimide as given in Methods, and the results are given in Table I. An endpoint of reaction was reached within 5 h for both α and β (Table I). The numbers obtained, 144 nmol/mg α subunit and 50 nmol/mg β subunit, are in agreement with the number of SH groups titrated with DTNB and with the labelling of the kidney enzyme, as recently reported [29]. This indicates that there is no labelling of amino groups by *N*-ethylmaleimide in α and β, as DTNB reacts only with SH groups. Both titrations are in agreement with published amino acid analysis data on the half-cystine content of the two chains [30]. The reaction of *N*-ethylmaleimide with γ did not reach an endpoint after 10 h of incubation (Table I). This is presumably due to the slow reaction of amino groups with *N*-ethylmaleimide. Correspondingly, the number of *N*-ethylmaleimide molecules incorporated per mg enzyme after solubilization with SDS is larger than the number of SH groups titrated with DTNB (Table I).

Reaction of native ATPase with DTNB and N-ethylmaleimide

The concentration of *N*-ethylmaleimide or DTNB necessary to modify the SH groups essential to enzyme activity in the case of shark rectal gland ($Na^+ + K^+$)-ATPase is much lower (Fig. 2) than in the brain [8], kidney [13] or electroplax [12] enzymes. It is seen that about 50 μM *N*-ethylmaleimide or DTNB is sufficient to reduce the activity to about 16% with 30 min of incubation at 37°C. A 20-fold increase in the reagent concentration did not change the extent of inactivation after 30 min of incubation very much, and at a reagent concentration of 5 mM about 3% of the activity remains after 30 min incubation. This indicates the existence of more than one class of SH groups in the ATPase, as the reaction with *N*-ethylmaleimide is irreversible. This prompted us to investigate the reaction mechanism of the reaction of *N*-ethylmaleimide with the ATPase.

Classification of SH groups

It was observed that the enzyme activity was retained when a sufficient amount of glycerol or sucrose (35% w/v) was present in the *N*-ethylmaleimide incubation medium (0.1 mM) (Fig. 3). This shows that glycerol and sucrose can induce a conformation of the enzyme in which the



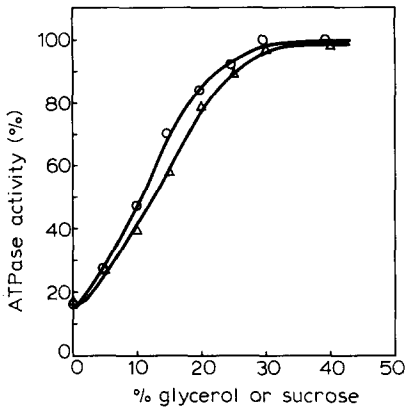


Fig. 2. (Left.) Concentration dependence of *N*-ethylmaleimide and DTNB on the inactivation of the $(Na^+ + K^+)$-ATPase. Enzyme (1 mg/ml) was incubated for 30 min under standard conditions (see Methods) with 150 mM K^+ present and *N*-ethylmaleimide (NEM) (★———★) or DTNB (○———○) as indicated. The phosphatase activity was lost in parallel to the ATPase activity (the ATPase/phosphatase ratio being constant about 6.4). The ATPase activity is given as percentage of control (no *N*-ethylmaleimide added).

Fig. 3. (Right.) The effect of glycerol and sucrose on the reaction with NEM. Enzyme was incubated under standard conditions for 30 min with 150 mM K^+ and 0.1 mM *N*-ethylmaleimide and glycerol (○———○) or sucrose (△———△) as indicated. Activity is given as percentage of control enzyme (no *N*-ethylmaleimide added) and is measured as described in Methods.

SH groups essential to activity are not reactive towards *N*-ethylmaleimide. Reaction with *N*-ethyl[^{14}C]maleimide (0.1 mM) in the presence of 40% glycerol, i.e., under conditions where the activity is retained, showed that two SH groups per α and one per β reacted with *N*-ethylmaleimide (see Table II in the following paper [35] for details). These six SH groups per $\alpha_2\beta_2$ are called Class I groups, and are not essential to enzyme activity.

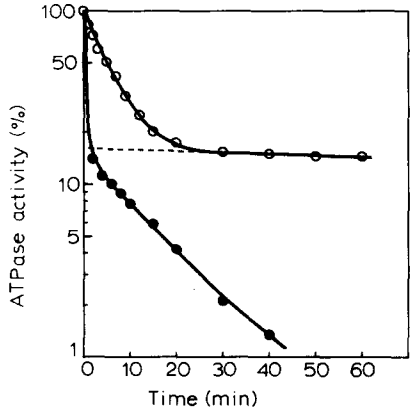


Fig. 4. Time course of inactivation with *N*-ethylmaleimide. $(Na^+ + K^+)$-ATPase was treated with 0.1 mM *N*-ethylmaleimide (○———○) or 5 mM *N*-ethylmaleimide (●———●) in the presence of 150 mM K^+ and 5% glycerol (see Methods). The $(Na^+ + K^+)$-ATPase activity is given as percentage of control (no *N*-ethylmaleimide added) on a logarithmic scale. The K^+-phosphatase activity was inhibited in parallel to the $(Na^+ + K^+)$-ATPase activity. See text for details of data fitting.

The time course of the inactivation of $(Na^+ + K^+)$-ATPase by 0.1 mM and 5 mM *N*-ethylmaleimide is shown in Fig. 4. With 0.1 mM *N*-ethylmaleimide the enzyme is activated only to about 16% of initial activity. A second addition of 0.1 mM *N*-ethylmaleimide after 30 min does not inactivate the enzyme further (not shown). This indicates that the SH-groups which can react with the low concentration of *N*-ethylmaleimide have all reacted. If the slow phase is subtracted from the rapid phase a single exponential decay of activity is obtained (Fig. 5). This shows that the SH groups reacted with 0.1 mM *N*-ethylmaleimide —leading to an enzyme with an activity of about 16%—react as a single class of groups. This class is called Class II and reacts with a second-order rate constant of 1.73 $mM^{-1} \cdot min^{-1}$. The number of SH groups in Class II is four per α (see the following paper [35] for details).

With 5 mM *N*-ethylmaleimide the Class II groups are readily reacted, and it is seen that the

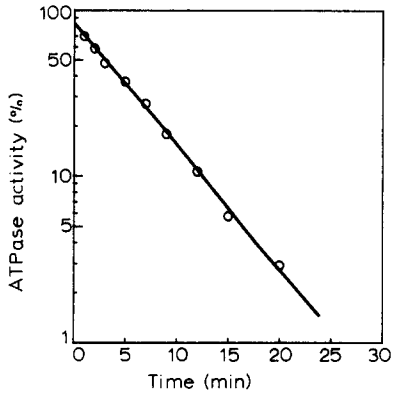


Fig. 5. Rapid-phase inactivation. The data from Fig. 4 with 0.1 mM *N*-ethylmaleimide are replotted after subtraction of the slow phase (dotted line in Fig. 4). The second-order rate constant obtained from this plot is 1.73 $mM^{-1} \cdot min^{-1}$.

enzyme activity can be completely abolished. The groups reacting with 5 mM *N*-ethylmaleimide—in addition to the Class II groups—are called Class III groups. Reaction of the Class III groups reduces the activity from about 16% to zero. The activity in this phase is also lost in a monoexponential fashion, Fig. 4. The data in Fig. 4 (with 5 mM *N*-ethylmaleimide) can be fitted by an expression:

$$A_t = A_0 \cdot \exp(-t \cdot k_2 \cdot N_0 \cdot \exp(-k_3 \cdot t))$$

where A_0 is the enzyme activity when the Class II groups have reacted (16% of initial), A_t is the activity at a given time and the second-order rate constant, k_2, is 0.0107 $mM^{-1} \cdot min^{-1}$. N_0 is the initial *N*-ethylmaleimide concentration, 5 mM, and the term $N_0 \cdot \exp(-k_3 \cdot t)$ provides for the decrease in the *N*-ethylmaleimide concentration with time because of hydrolysis and reaction with the histidine buffer used. The pseudo-first-order rate constant for this reaction, k_3, is 0.0135 min^{-1}. ($t_{1/2}$ is about 52 min, which shows that it is unnecessary to include this correction for the reaction with the Class II groups.)

The finding that there are two classes of SH groups with highly different reactivities towards *N*-ethylmaleimide suggests that either the enzyme preparation contains two different types of ($Na^+ + K^+$)-ATPase with widely different reactivities towards *N*-ethylmaleimide or that the enzyme is changed into another enzyme species with only 16% activity when the Class II groups are reacted and that the reaction of this enzyme species with *N*-ethylmaleimide at high concentrations—that is, the reaction of the Class III groups—leads to a completely inactive enzyme. It is not possible from the present experiments to formulate a complete model for the reaction of *N*-ethylmaleimide with the ($Na^+ + K^+$)-ATPase.

Effect of DTNB and N-ethylmaleimide

N-Ethylmaleimide and DTNB have different effects on kidney ($Na^+ + K^+$)-ATPase and it has been proposed that there are two classes of essential SH groups [15]. We have investigated whether *N*-ethylmaleimide and DTNB also have different effects on the shark ($Na^+ + K^+$)-ATPase. Fig. 6 shows the effect of protecting the Class II groups with DTNB before reaction with *N*-ethylmaleimide. Firstly it is seen that the enzyme can be reactivated with dithiothreitol after the reaction with DTNB (no *N*-ethylmaleimide). As expected, the reaction of enzyme with *N*-ethylmaleimide (no DTNB) cannot be reversed with dithiothreitol, and no reactivation occurs. However, when the Class II groups have been reacted with DTNB before addition of *N*-ethylmaleimide, it is still possible to

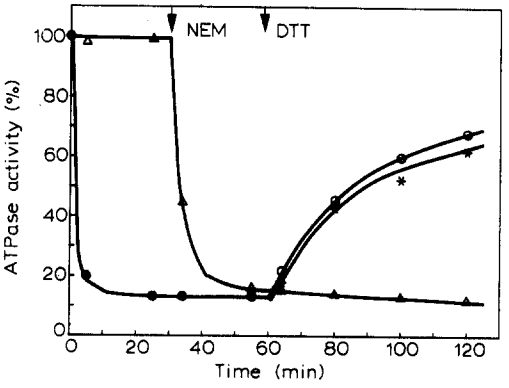


Fig. 6. The effect of a sequential addition of *N*-ethylmaleimide and DTNB. Enzyme was incubated under standard conditions with 150 mM K^+ present. Reaction was initiated by addition of 0.1 mM DTNB (○, ∗) or H_2O (△). After 30 min 0.2 mM *N*-ethylmaleimide (NEM) was added to one of the samples containing DTNB (○——○) and to the sample without DTNB (△——△). After another 30 min all samples were made 10 mM in dithiothreitol (DTT), and the ($Na^+ + K^+$)-ATPase activity was followed with time. Activity is given as percentage of control without DTNB or *N*-ethylmaleimide.

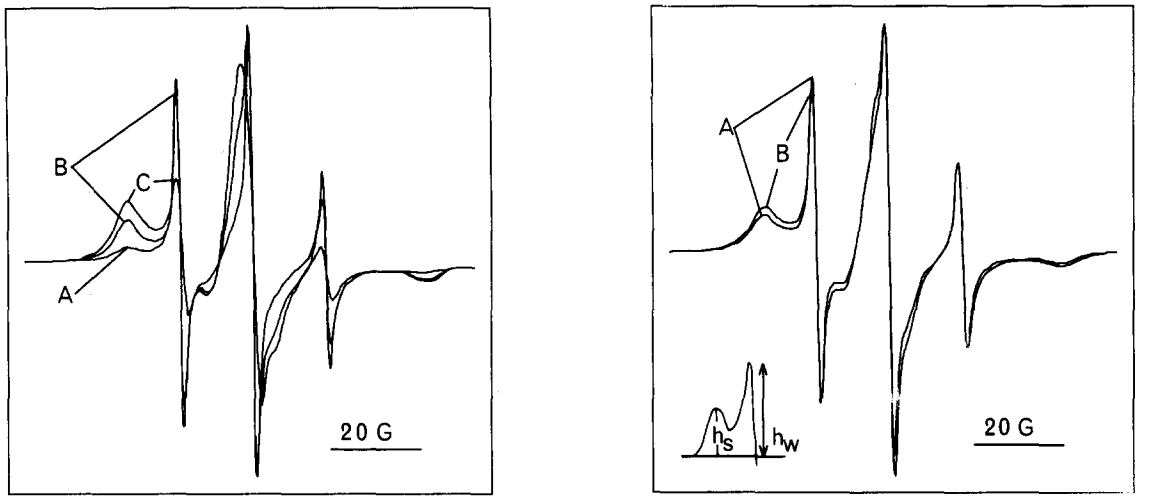


Fig. 7. ESR spectra of MSL-labelled $(Na^+ + K^+)$-ATPase. $(Na^+ + K^+)$-ATPase was treated with 0.1 mM MSL as given in Methods in the presence (A) or absence (B) of 40% glycerol, leading to labelling of Class I groups (A) or Class I+II groups (B). After a pretreatment with 0.1 mM *N*-ethylmaleimide in the presence of 40% glycerol, the Class II groups were labelled with 0.1 mM MSL in the absence of glycerol (C). The samples for ESR spectroscopy contained 10–14 mg protein ml in a 20 mM histidine buffer (pH 7.4). The $(Na^+ + K^+)$-ATPase activity (in μmol/mg per h) was 1307 for sample A, 251 for sample B and 238 for sample C.

Fig. 8. Effect of Na^+ and K^+ on Class I groups. $(Na^+ + K^+)$-ATPase was treated with MSL in the presence of 40% glycerol as given in Methods. The pellet was resuspended in 20 mM histidine (pH 7.4) with 150 mM K^+ (A) or Na^+ (B) present. The $(Na^+ + K^+)$-ATPase activity was 1250 μmol/mg per h. The parameters h_s and h_w are shown in the figure.

reactivate enzyme with dithiothreitol. This shows that the Class II SH groups of the shark enzyme

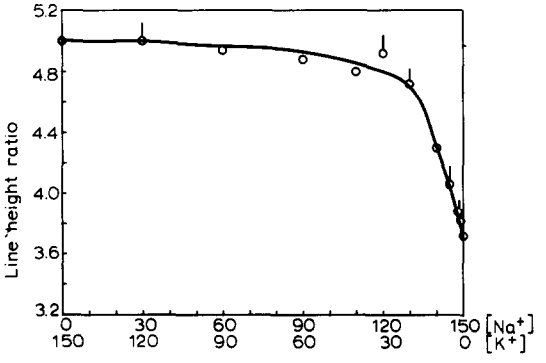


Fig. 9. Effect of Na^+ and K^+ on the ESR line heights. $(Na^+ + K^+)$-ATPase was prepared as given in the legend to Fig. 8, and the resulting pellet was resuspended in Na^+ and K^+ at different ratios, keeping the cation concentration at 150 mM. The line heights h_s and h_w (see Fig. 8) were measured and the ratio h_w/h_s is given as a function of the ligand combination. The data given are means of three experiments and the S.E. is indicated in the figure.

which react with DTNB are the same as those reacting with *N*-ethylmaleimide, in contrast to the behaviour of the kidney enzyme [15].

The reduction potential of the Class II SH groups must be very favourable for reaction with DTNB, as a 10-fold excess of mercaptoethanol over DTNB in the preincubation mixture did not alter the inactivation with DTNB (not shown). It is also seen (Fig. 6) that the time needed for reactivation with 18 mM dithiothreitol is very long. The conformation of the protein around the reactive SH groups must somehow prevent the bound TNB from leaving the enzyme.

Spin-labelling of the SH groups

The SH groups of the $(Na^+ + K^+)$-ATPase were further characterized by the use of a spin-labelled maleimide derivative, MSL. MSL inactivates the $(Na^+ + K^+)$-ATPase in the same manner as *N*-ethylmaleimide (not shown). Fig. 7 shows the ESR spectra of enzyme where the Class I groups have been reacted (A), where both Class I and Class II groups have been reacted (B), and where only

Class II groups have been reacted (C) with MSL. All spectra display two components: a strongly immobilized component (S) and a weakly immobilized component (W). The major difference between the spectra A and B is the larger amount of strongly immobilized label in B. This suggests that the spin labels of the Class II groups are mostly strongly immobilized. This is confirmed by spectrum C, where the Class I groups have been reacted with *N*-ethylmaleimide (without spin label) and the Class II groups have been reacted with MSL (see legend to Fig. 7). The Class II groups, which are important for full enzyme activity, must be located in an environment where motion is quite restricted; that is, not on the surface of the protein. Spectrum C also gives an indication that not all the Class II groups have the same environment.

This shows that the Class II groups—although they react with *N*-ethylmaleimide as a homogeneous class (Fig. 5)—are not all identical with respect to their microenvironments (see also the following paper).

Effects of Na^+ and K^+ on spin-labelled ($Na^+ + K^+$)-ATPase

Spin label attached to the Class I groups can monitor the difference between the conformations seen with Na^+ and K^+, Fig. 8. The enzyme is labelled with 0.1 mM MSL in the presence of 40% glycerol and washed by repeated centrifugations (see Methods). The pellet is resuspended in 150 mM Na^+ or K^+ or a combination of these ions (see Figs. 8 and 9). Fig. 8 shows that the ratio of the heights of the weakly and the strongly immobilized components (h_w/h_s) is 4.9 for the K^+ form and 3.8 for the Na^+ form, a 20% difference. This difference suggests that the weakly immobilized groups become more immobilized when the enzyme is transferred from the K^+ form to the Na^+ form.

Fig. 9 shows the ratio h_w/h_s for different combinations of Na^+ and K^+, keeping the total cation concentration constant at 150 mM. The Na^+/K^+ ratio giving half-maximal change is 137/13, which is in agreement with the observations on the effect of Na^+ and K^+ on tryptic digestion of kidney enzyme [31].

Discussion

1. ($Na^+ + K^+$)-ATPase from rectal glands of *S. acanthias* contains 34 SH groups and two disulphide bridges per $\alpha_2\beta_2$ unit, in agreement with the SH content of the kidney enzyme [15]. 15 of the SH groups are located on the α subunit and the β subunit contains two SH groups as well as the disulphide bridge. Titrations with DTNB and *N*-ethylmaleimide are in good agreement, which shows that the groups reacted with *N*-ethylmaleimide are SH groups. The finding that half of the cysteines on the β subunit form disulphide bridges explains why the amount of $\alpha\beta$ crosslinking with sulphydryl reagents is much smaller than the amount of $\alpha\alpha$ crosslinking, although the half-cystine content of the two chains is about the same on a molar basis [30,32].

No attempt has been made to determine the extinction coefficient for the calibration of the Lowry method [22] with quantitative amino acid analysis. This could change the figures in the stoichiometries somewhat (see Refs. 33 and 34 for a discussion of this).

2. It has been shown that 2–6 SH groups react with *N*-ethylmaleimide before inactivation occurs [16,29]. In this paper it is shown that these groups (Class I) can be modified selectively in the presence of glycerol or sucrose with no loss of activity. It is also shown that a spin probe bound to these groups can monitor the difference between the Na^+ and K^+ forms on a fully active enzyme. The Class I groups are located predominantly on the surface of the protein, which may explain why they react most rapidly [16,29].

3. The inactivation of the enzyme with *N*-ethylmaleimide occurs in a biphasic manner (Fig. 4). About 84% of the enzyme activity is lost with low concentrations of *N*-ethylmaleimide (0.1 mM) and the rest of the activity can be completely abolished by high concentrations of *N*-ethylmaleimide (5 mM). This suggests two classes of SH groups (Class II and III) essential to activity. This is in contrast to the behaviour of the kidney enzyme [15,16], in which the activity is lost in a monoexponential fashion, but in agreement with the biphasic inactivation of the brain enzyme [8]. The K^+-phosphatase activity is lost in parallel to

the $(Na^+ + K^+)$-ATPase activity, in agreement with the findings of Schoot et al. [15]. It was not possible to obtain a further classification by a sequential modification as DTNB and *N*-ethylmaleimide react with the same Class II groups (Fig. 6). It should be noted that Schoot et al. [15] react the enzyme with DTNB and *N*-ethylmaleimide in the absence of cations, which might account for the discrepancy between the results with rectal gland and kidney enzyme. ESR spectroscopy showed that the Class II groups are in a part of the protein molecule where motion is quite restricted. These SH groups may serve as probes for the measurement of rotational correlation times of the protein by saturation transfer ESR spectroscopy (work in progress).

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