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

XLII. EVIDENCE FOR TWO CLASSES OF ESSENTIAL SULFHYDRYL GROUPS

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Summary

1. Preincubation of purified (Na^++K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparations from rabbit kidney outer medulla with 5,5′-dithiobis-(2-nitrobenzoic acid) inhibits the (Na^++K^+)-ATPase and K^+-stimulated 4-nitrophenylphosphatase activities. Phosphorylation of the enzyme by ATP and the Na^+-stimulated ATPase activity are inhibited to the same extent as the (Na^++K^+)-ATPase activity, whereas the K^+-stimulated 4-nitrophenylphosphatase activity is inhibited much less.

2. Titration with 5,5′-dithiobis-(2-nitrobenzoic acid) in sodium dodecyl sulphate shows the presence of 36 reactive sulfhydryl groups per molecule (Na^++K^+)-ATPase (M_r = 250 000).

3. Treatment with *N*-ethylmaleimide, resulting in complete inhibition of (Na^++K^+)-ATPase activity, leads to modification of 26 sulfhydryl groups, whereas treatment with 5,5′-dithiobis-(2-nitrobenzoic acid) results in modification of 12 sulfhydryl groups under the same conditions.

4. The reaction of *N*-ethylmaleimide with an essential SH-group is not prevented by previous blocking of sulfhydryl groups with 5,5′-dithiobis-(2-nitrobenzoic acid).

5. These findings indicate the existence of at least two classes of sulfhydryl groups on the enzyme, each containing at least one vital group. The difference between these classes consists in their different reactivity towards 5,5′-dithiobis-(2-nitrobenzoic acid) and *N*-ethylmaleimide.

Introduction

The enzyme (Na^++K^+)-ATPase (ATP phosphohydrolase, EC 3.1.6.3) is involved in the transport of Na^+ and K^+ across the plasma membrane [1—5]. Pre-

Abbreviation: CDTA, *trans*-1,2-diamino cyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

viously it has been shown that blocking of one or more of the sulfhydryl groups of the enzyme leads to loss of activity [6]. One of these groups appears to be located inside the ATP binding center [7]. In a preceding paper [8] we have presented evidence that there are also one or more vital sulfhydryl groups located outside the catalytic center.

Since the reactivity of the vital sulfhydryl groups seems to depend on the conformation of the enzyme [9,10], the study of the behaviour of these groups can help to elucidate the structure-function relationships of the enzyme.

In the present study we have determined the total number of free sulfhydryl groups in the enzyme by titration with the specific sulfhydryl reagent 5,5'-dithiobis-(2-nitrobenzoic acid), and the fraction exposed under native conditions. The use of this reagent in combination with *N*-ethylmaleimide allows us to differentiate between two classes of sulfhydryl groups, each containing at least one vital group.

Materials and Methods

Enzyme preparation. Purified $(Na^+ + K^+)$-ATPase is obtained from rabbit kidney outer medulla microsomes by extraction with sodium dodecyl sulphate and continuous sucrose density gradient centrifugation, as described by Jørgensen [11].

The highly active $(Na^+ + K^+)$-ATPase preparation thus obtained is incubated for 30 min at 37°C in a medium containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$ and 25 mM imidazole · HCl (pH 7.4) to remove ATP and is then centrifuged for 10 min at 300 000 × *g*. The resulting pellet is washed twice by resuspension and centrifugation in 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5). The preparation is stored at −20°C in a buffer containing 250 mM sucrose, 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5). The specific $(Na^+ + K^+)$-ATPase activity amounts to 1000—2000 μmol P_i/mg protein per h, without ouabain-insensitive ATPase activity. The specific K^+-stimulated, ouabain-inhibited 4-nitrophenylphosphatase activity is 200—400 μmol 4-nitrophenol/mg protein per h. No decrease in specific activity is observed after incubation of the enzyme for 60 min at 37°C in a buffer containing 25 mM imidazole · HCl (pH 7.5), 2 mM CDTA.

Enzyme assays. $(Na^+ + K^+)$-ATPase activity is determined as the difference in $^{32}P_i$ production at 37°C in a medium containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 30 mM imidazole · HCl (pH 7.4) and the same medium without 10 mM KCl, but with addition of 0.1 mM ouabain. The P_i production is measured either by means of reaction with molybdate-$FeSO_4$ and reading the absorbance of the reduced phospho-molybdate complex, or by a radioactive procedure in which 1 μm [γ-^{32}P]ATP is added to the above reaction mixture and the $^{32}P_i$-molybdate complex is extracted into isobutanol and counted for radioactivity.

Na^+-stimulated ATPase activity is measured as the difference in P_i production at 37°C in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 μM [γ-^{32}P]ATP, 30 mM imidazole · HCl (pH 7.4) and a medium containing in addition 0.1 mM ouabain.

K^+-stimulated 4-nitrophenylphosphatase activity is determined as the difference in 4-nitrophenol production at 37°C in a medium containing 10 mM KCl,

6 mM $MgCl_2$, 5 mM 4-nitrophenyl phosphate, 1 mM CDTA, 30 mM imidazole · HCl (pH 7.4) and a medium of the same composition but without 10 mM KCl and with 0.1 mM ouabain.

Phosphorylation by ATP is carried out in a medium containing 100 mM NaCl, 5 mM MgCl, 20 μM [γ-^{32}P]ATP, 30 mM imidazole · HCl (pH 7.4) and 50—250 μg protein/ml. After 15 s at 0° C the phosphorylation is stopped by addition of 5% trichloroacetic acid in 0.1 M H_3PO_4. The denatured protein is isolated by filtration on a Selectron AE 95 filter (1.2 μm pore size, Schleicher and Schüll, Dassel, G.F.R.). The washed filter is counted for radioactivity.

Further details of the enzyme assays are given in ref. 8.

Reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). Incubation with 5,5'-dithiobis-(2-nitrobenzoic acid) is performed, unless otherwise stated, at 37°C in a medium containing 25 mM imidazole · HCl, 2 mM CDTA, 100 μg protein/ml and 5,5'-dithiobis-(2-nitrobenzoic acid) at the stated concentrations, at pH 7.5 for 60 min. The reaction is stopped by 100-fold dilution with 25 mM imidazole · HCl, 2 mM CDTA (pH 7.5). The diluted reaction mixture is kept on ice, and aliquots are used within minutes for assay of enzyme activities.

When additives present during preincubation with 5,5'-dithiobis-(2-nitrobenzoic acid) (e.g. KCl, 4-nitrophenyl phosphate, ATP) may interfere with the subsequent assay, the preparation is first subjected to column chromatography. The reaction mixture (100 μl) is placed on a column (Sephadex G-25 coarse, 100 × 5 mm), equilibrated in 25 mM imidazole · HCl, 2 mM CDTA (pH 7.5). The enzyme is eluted in a 1000 μl fraction, free of contaminants, after 900 μl elution with equilibration buffer.

Reaction with N-ethylmaleimide. Incubation with *N*-ethylmaleimide is performed for 30 min at 37°C in a medium containing 25 mM imidazole · HCl (pH 7.5), 2 mM CDTA, 0.01—4 mg protein/ml and *N*-ethylmaleimide at the stated concentration. After incubation excess *N*-ethylmaleimide is removed either by addition of excess 1,4-dithioerythritol or by gel filtration over a Sephadex G-25 column as described above.

Determination of sulfhydryl groups with 5,5'-dithiobis-(2-nitrobenzoic acid). Sulfhydryl groups are determined by reading the 412 nm absorbance of 5-thio-2-nitrobenzoic acid, released during reaction of the enzyme with the parent reagent. A mixture of 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 400 μg protein/ml, 25 mM imidazole · HCl (pH 7.5), with or without 1% (w/w) sodium dodecyl sulphate, is incubated for 60 min at 37°C. Thereafter insoluble protein is removed by centrifugation (15 min at 20 000 × *g*) and the 412 nm absorbance of the supernatant is determined. The absorbance of 5-thio-2-nitrobenzoic acid is calibrated by adding known amounts of cysteine · HCl to the reaction mixture instead of enzyme. Corrections are made for imidazole and protein absorbance. The number of sulfhydryl groups determined in the presence of 1% sodium dodecyl sulphate is referred to as "total sulfhydryl groups".

The number of sulfhydryl groups per molecule ($Na^+ + K^+$)-ATPase is calculated by assuming a molecular weight of 250 000 [12].

Determination of the partition coefficients of 5,5'-dithiobis-(2-nitrobenzoic acid) and N-ethylmaleimide. The partition coefficient of *N*-ethylmaleimide is determined in the following way: 1 ml 10 mM *N*-ethylmaleimide in 25 mM imidazole · HCl (pH 7.5) is mixed with 10 ml water-saturated octanol-1. After

separation of the two phases, the *N*-ethylmaleimide concentration is determined in aliquots of both phases. The aqueous layer aliquot is diluted 100-fold and mixed with 0.1 volume 100 μM cysteine · HCl, 25 mM imidazole · HCl (pH 7.5). The octanol layer aliquot is mixed with two volumes of the cysteine solution. After incubation for 60 min at 37°C, remaining cysteine is determined by addition of an equal volume of 5 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and further incubation for 60 min at 37°C. The 412 nm absorbance is determined in the water layer. The concentration of *N*-ethylmaleimide can be calculated from the decrease in the amount of cysteine.

The partition coefficient for 5,5′-dithiobis-(2-nitrobenzoic acid) is determined in a similar way, except that five volumes octanol layer are mixed with one volume of the cysteine solution. Calibration curves are obtained by reading the 412 nm absorbance of solutions of known concentrations of cysteine and excess 5,5′-dithiobis-(2-nitrobenzoic acid) in 25 mM imidazole (pH 7.5) buffer.

Protein determinations. Protein determinations are performed according to Lowry et al. [13] after trichloroacetic acid precipitation as described by Jørgensen [11].

Materials. ATP is obtained from Boehringer (Mannheim, W. Germany), *N*-ethylmaleimide and 4-nitrophenyl phosphate (disodium salt) from Merck (Darmstadt, W. Germany). 4-Nitrophenyl phosphate is converted to the imidazole salt by ion-exchange chromatography over a Dowex 50 column (H^+ form) and subsequent neutralization with imidazole. 5,5′-Dithiobis-(2-nitrobenzoic acid) is purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), [γ-^{32}P]-ATP (3.0 Ci/mmol initial specific radioactivity) from the Radiochemical Center (Amersham, England).

All other chemicals are of reagent grade.

Results

Effect of 5,5′-dithiobis-(2-nitrobenzoic acid) on (Na^++K^+)-ATPase activity

Preincubation of purified (Na^++K^+)-ATPase with 5,5′-dithiobis-(2-nitrobenzoic acid) causes inactivation of the (Na^+ + K^+)-ATPase activity. The inhibition follows pseudo first-order kinetics (Fig. 1a), as is also the case for the inhibition by *N*-ethylmaleimide (not shown). By plotting the logarithm of the time for 50% inactivation against the logarithm of the inhibitor concentration, the number of molecules 5,5′-dithiobis-(2-nitrobenzoic acid) reacting per enzyme molecule can be calculated from the slope of this curve [14]. This number appears to be 1.0, which indicates true second-order kinetics for the ATPase inhibition by 5,5′-dithiobis-(2-nitrobenzoic acid) (Fig. 1b), as is also the case for *N*-ethylmaleimide.

When preincubation is carried out for 60 min at 37°C in a medium containing 100 μg protein/ml, 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5), the enzyme is completely inhibited by 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 50% inhibited by 20—50 μM of this substance (Fig. 2). The rate of inhibition increases at increasing pH (Fig. 3). Reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) is specific for sulfhydryl groups [15].

Addition of ATP to the preincubation medium antagonizes the inhibitory effect of 5,5′-dithiobis-(2-nitrobenzoic acid) (Fig. 4), but only under conditions

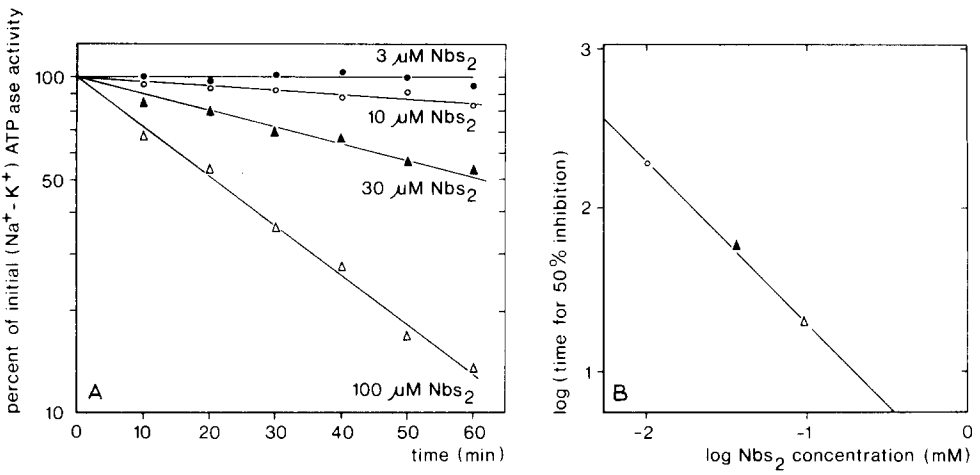

Fig. 1. (A) Time course for the inhibition of $(Na^+ + K^+)$-ATPase activity by 5,5′-dithiobis-(2-nitrobenzoic acid). The reaction mixture for treatment with 5,5′-dithiobis-(2-nitrobenzoic acid) (abbreviated: Nbs_2) contains 100 μg protein/ml, 25 mM imidazole · HCl (pH 7.5) 2 mM CDTA and various concentrations of 5,5′-dithiobis-(2-nitrobenzoic acid) (●, $3 \cdot 10^{-3}$ mM; ○, 10^{-2} mM, ▲, $3 \cdot 10^{-2}$ mM; △, 10^{-1} mM). After incubation (at 37°C) the reaction mixture is diluted 30-fold, and assayed for $(Na^+ + K^+)$-ATPase activity as described under Materials and Methods. (B) Graph of log (time for 50% inhibition) against log (inhibitor concentration). Meaning of symbols as in A.

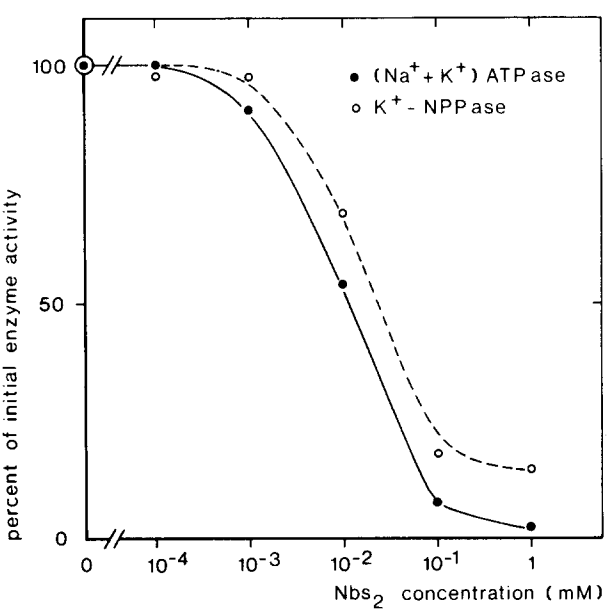

Fig. 2. Inhibition of $(Na^+ + K^+)$-ATPase and K^+-stimulated 4-nitrophenylphosphatase activities by 5,5′-dithiobis-(2-nitrobenzoic acid). Reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (abbreviated: Nbs_2) is carried out in a medium containing: 100 μg protein/ml, 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5) and 5,5′-dithiobis-(2-nitrobenzoic acid) at the stated concentrations. After 60 min incubation at 37°C the reaction mixture is diluted 30-fold, and aliquots are assayed for $(Na^+ + K^+)$-ATPase and K^+-stimulated 4-nitrophenyl-phosphatase activities as described under Materials and Methods.

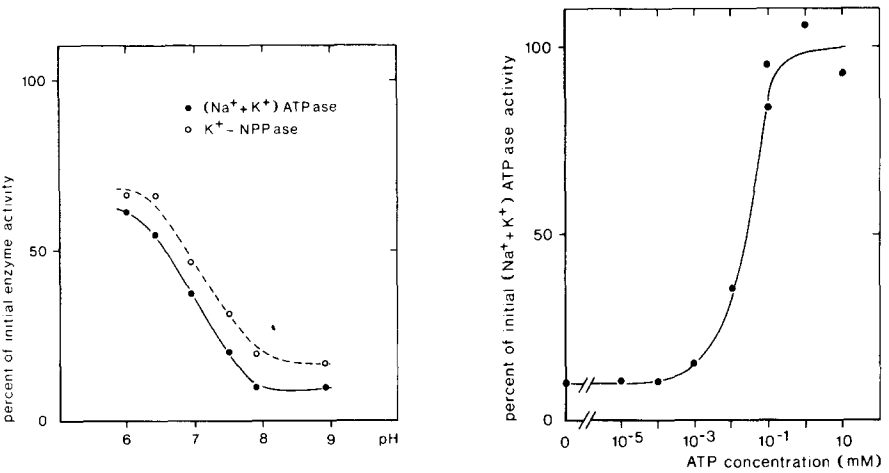

Fig. 3. Effect of pH on inhibition by 5,5′-dithiobis-(2-nitrobenzoic acid). The pre-incubation medium contains: 100 μg protein/ml, 60 μM 5,5′-dithiobis-(2-nitrobenzoic acid), 2 mM CDTA and 50 mM Tris/maleate buffer at the indicated pH values. After 60 min reaction at 37°C, the inhibition is stopped by 30-fold dilution in 25 mM imidazole · HCl (pH 7.5). Aliquots are assayed for $(Na^+ + K^+)$-ATPase and K^+-stimulated 4-nitrophenylphosphatase (K^+-NPPase) activities after pre-incubation with 5,5′-dithiobis-(2-nitrobenzoic acid). Corrections are made for the spontaneous inactivation of the enzyme at the given pH.

Fig. 4. Effect of ATP on inhibition of $(Na^+ + K^+)$-ATPase activity by 5,5′-dithiobis-(2-nitrobenzoic acid). The reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) is carried out for 60 min at 37°C in a medium containing 100 μg protein/ml, 240 μM 5,5′-dithiobis-(2-nitrobenzoic acid), 25 mM imidazole · HCl (pH 7.5), 2 mM CDTA and ATP (Tris salt) in the indicated concentrations. The reaction mixture is diluted 100-fold, and aliquots are assayed for $(Na^+ + K^+)$-ATPase activity as described under Materials and Methods.

TABLE I

EFFECT OF VARIOUS LIGANDS ON INHIBITION BY 5,5′-DITHIOBIS-(2-NITROBENZOIC ACID)

Treatment with 5,5′-dithiobis-(2-nitrobenzoic acid) is performed in a medium containing: 240 μM 5,5′-dithiobis-(2-nitrobenzoic acid) (except for the control), 25 mM imidazole · HCl (pH 7.5), 100 μg protein/ml and various ligands, as indicated in the table, for 60 min at 37°C. After reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) the ligands are removed by gel filtration on a Sephadex G-25 column, and aliquots are assayed for K^+-stimulated 4-nitrophenylphosphatase (K^+-NPPase) and $(Na^+ + K^+$ATPase) activity as described under Materials and Methods. Results from two experiments, carried out in duplicate, are presented as means with standard errors.

Ligands added during 5,5′-dithiobis-(2-nitrobenzoic acid reaction	Residual acitivity	
	K^+-NPPase (%)	$(Na^+ + K^+)$-ATPase (%)
Control (no 5,5′-dithiobis-(2-nitrobenzoic acid))	≡100	≡100
4 mM CDTA	12 ± 1.5	1.2 ± 1.2
10 mM ATP + 4 mM CDTA	98 ± 10	75 ± 5
10 mM ATP + 100 mM NaCl + 5 mM $MgCl_2$	64 ± 8	8 ± 2.4
10 mM 4-nitrophenyl phosphate + 4 mM CDTA	23 ± 1	4.9 ± 0.2
10 mM 4-nitrophenyl phosphate + 5 mM $MgCl_2$	26 ± 5	2.8 ± 0.1
10 mM 4-nitrophenyl phosphate + 5 mM $MgCl_2$ + 10 mM KCl	36 ± 9	4.0 ± 0.6

where no phosphorylation can occur, i.e. in the absence of Mg^{2+} and Na^+ (Table I). Half-maximal protection is obtained with 30 μM ATP (Fig. 4). Addition of 4-nitrophenyl phosphate has no effect on the inhibition by 5,5'-dithiobis-(2-nitrobenzoic acid).

Effects of 5,5'-dithiobis-(2-nitrobenzoic acid) on partial reactions of the ($Na^+ + K^+$)-ATPase system

Treatment with 5,5'-dithiobis-(2-nitrobenzoic acid) also inhibits the partial reactions of the ($Na^+ + K^+$)-ATPase activity. The Na^+-stimulated ATPase activity, which is apparent at micromolar ATP concentrations [16,17], is inhibited to the same extent as the (Na^++K^+)-ATPase activity (69 ± 6.7 vs. 69 ± 4.6% inhibition, 3 experiments). The same is the case for the phosphorylation by ATP in the presence of Na^+ and Mg^{2+} (Fig. 5).

The K^+-stimulated 4-nitrophenylphosphatase activity is also inhibit after treatment of the enzyme with 5,5'-dithiobis-(2-nitrobenzoic acid), but to a lesser extent than the (Na^++K^+)-ATPase activity. This is true at various concentrations of the reagent (see Fig. 2), as well as at different pH values of the preincubation medium (see Fig. 3). Addition of ATP to the preincubation medium antagonizes the inhibitory effect of 5,5'-dithiobis-(2-nitrobenzoic acid) on the 4-nitrophenylphosphatase activity, primarily under non-phosphorylating conditions but also under phosphorylating conditions (Table I). 4-Nitrophenyl phosphate partially prevents the inhibition under phosphorylating conditions, i.e. in the presence of Mg^{2+} with or without Na^+, as well as under non-phosphorylating conditions (Table I).

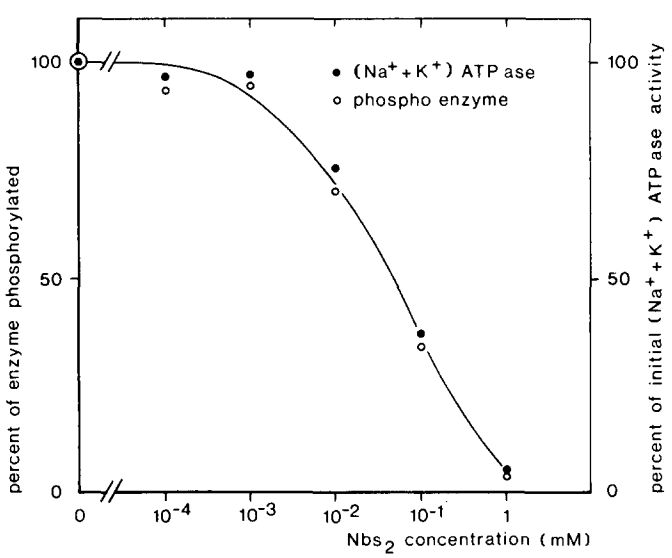

Fig. 5. Inhibition of phosphorylation and (Na^++K^+)-ATPase activity by 5,5'-dithiobis-(2-nitrobenzoic acid). Pre-incubation with 5,5'-dithiobis-(2-nitrobenzoic acid) (abbreviated: Nbs_2) is carried out for 60 min at 37°C in a medium containing: 200 μg protein/ml, 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5), and 5,5'-dithiobis-(2-nitrobenzoic acid) at the indicated concentrations. The pre-incubation is ended by 4-fold dilution in the appropriate phosphorylation buffer, and phosphorylation (○) and (Na^++K^+)-ATPase activity (•) are assayed as described under Materials and Methods.

TABLE II

EFFECT OF 5,5'-DITHIOBIS-(2-NITROBENZOIC ACID) ON INHIBITION BY *N*-ETHYLMALEIMIDE

Pre-incubation I is carried out in a medium containing: 0 or 240 μM 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs_2) imidazole · HCl (pH 7.5), 2 mM CDTA, 100 μg protein/ml at 37°C for 60 min. The mixture is then applied to a Sephadex G-25 column, to remove 5,5'-dithiobis-(2-nitrobenzoic acid). Preincubation II is performed in a medium containing: 0 or 10 mM *N*-ethylmaleimide, 0 or 10 mM ATP, 25 mM imidazole · HCl (pH 7.5), 4 mM CDTA, 10 μg protein/ml for 30 min at 37°C. Preincubation III is performed with 10 mM dithioerythritol for 30 min at 37°C. Aliquots are asssayed for ($Na^+ + K^+$)-ATPase activity. Results are presented as means with standard error for *n* experiments.

Additives during pre-incubation			Residual activity ($Na^+ + K^+$)-ATPase (%)	(*n*)
I	II	III		
—	—	dithioerythritol	100	5
Nbs_2	—	—	31 ± 3	4
Nbs_2	—	dithioerythritol	75 ± 7	5
Nbs_2	*N*-ethyl-maleimide	dithioerythritol	22 ± 4	5
Nbs_2	*N*-ethyl-maleimide + ATP	dithioerythritol	52 ± 6	2

Effect of subsequent treatment with N-ethylmaleimide

To investigate whether 5,5'-dithiobis-(2-nitrobenzoic acid) reacts with the same functional groups as *N*-ethylmaleimide does, we have applied these reagents consecutively and have used 1,4-dithioerythritol to reverse the reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). The inhibitory effect of *N*-ethylmaleimide cannot be reversed by subsequent treatment with dithioerythritol [8].

Incubation with 1,4-dithioerythritol after preincubation with 5,5'-dithiobis-(2-nitrobenzoic acid) leads to reversal of the inhibition caused by 5,5'-dithiobis-(2-nitrobenzoic acid) (Table II). When after preincubation with 5,5'-dithiobis-(2-nitrobenzoic acid) the enzyme is treated with *N*-ethylmaleimide, incubation with 1,4-dithioerythritol does not restore the original activity. This suggests that *N*-ethylmaleimide can react with additional vital groups in the enzyme complex after certain essential sulfhydryl groups have previously been blocked with 5,5'-dithiobis-(2-nitrobenzoic acid). Table II also shows that in this case the effect of *N*-ethylmaleimide can be antagonized, at least in part, by addition of 10 mM ATP.

Number of sulfhydryl groups reacting with 5,5'-dithiobis-(2-nitrobenzoic acid) and N-ethylmaleimide

We have determined the number of sulfhydryl groups able to react with 5,5'-dithiobis-(2-nitrobenzoic acid) in completely inactivating concentration (1 mM) after preincubation with various concentrations of *N*-ethylmaleimide. This titration is carried out in the presence or absence of 1% (w/w) sodium dodecyl sulphate. The total number of sulfhydryl groups titrable per molecule of the (Na^++K^+)-ATPase complex (M_r = 250 000) in the presence of sodium dodecyl sulphate is 36 ± 2.0. Without sodium dodecyl sulphate 5,5'-dithiobis-(2-nitrobenzoic acid) reacts with 12 ± 1.4 of these sulfhydryl groups.

TABLE III

SULFHYDRYL GROUPS AFTER *N*-ETHYLMALEIMIDE INHIBITION

Treatment with *N*-ethylmaleimide is carried out in a medium containing *N*-ethylmaleimide (0, 1 or 10 mM), 25 mM imidazole · HCl (pH 7.5), 2 mM CDTA and 4 mg protein/ml, for 30 min at 37°C. Excess *N*-ethylmaleimide is removed by gel filtration on a Sephadex G-25 column. Aliquots of the eluate are assayed for $(Na^+ + K^+)$-ATPase activity and sulfhydryl group content, as indicated under Materials and Methods. Results are the means with standard errors of three experiments, except for the value of 36 ± 2.0 sulfhydryl groups, which represents eight determinations. Mean specific activity of the preparations used is 1336 (S.E. 140) μmol ATP splitted/mg protein per h.

Concentration *N*-ethylmaleimide during preincubation (mM)	Residual $(Na^+ + K^+)$-ATPase activity (%)	Free sulfhydryl groups per mol enzyme	
		No SDS	1% SDS
0	≡100	12 ± 1.4	36 ± 2.0
1	55 ± 5	6 ± 0.7	25 ± 3.5
10	3.3 ± 0.4	0	10 ± 1.7

When the enzyme has previously been treated with 10 mM *N*-ethylmaleimide, a nearly completely inhibitory concentration, 5,5′-dithiobis-(2-nitrobenzoic acid) does not react with the enzyme in the absence of sodium dodecyl sulphate. In the presence of the latter substance, 5,5′-dithiobis-(2-nitrobenzoic acid) reacts with 10 ± 1.7 of the 36 sulfhydryl groups, suggesting that *N*-ethylmalemide has reacted with 36 − 10 = 26 sulfhydryl groups. Intermediate results are obtained when the enzyme is preincubated with 1 mM instead of 10 mM *N*-ethylmaleimide (Table III).

In summary: of the 36 sulfhydryl groups per enzyme molecule minimally 26 react with *N*-ethylmaleimide, whereas maximally 10 do not react. From these 26 residues at least 12 can also be modified by 5,5′-dithiobis-(2-nitrobenzoic acid). At least one residue of the latter 12, and at least one of the 14 other residues is vital for the activity of the enzyme. The reaction between each of these residues and the respective sulfhydryl reagents can be prevented by ATP.

Partition coefficients of N-ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid)

The partition coefficients of *N*-ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid) between 25 mM imidazole (pH 7.5) and octanol-1 have been determined as described under Materials and Methods. The observed values are: 15 for *N*-ethylmaleimide (buffer/octanol) and $5 \cdot 10^3$ for 5,5′-dithiobis-(2-nitrobenzoic acid) (buffer/octanol). The latter value agrees with the value of $4 \cdot 10^3$ reported by Murphy [18]. These values indicate that *N*-ethylmaleimide is much more lipophilic than 5,5′-dithiobis-(2-nitrobenzoic acid).

Discussion

$(Na^+ + K^+)$-ATPase, isolated from rabbit kidney outer medulla, can be inactivated with the sulfhydryl reagents 5,5′-dithiobis-(2-nitrobenzoic acid) and *N*-ethylmaleimide. In both cases the inhibition reaction obeys second-order kinetics, indicating that inhibition results from the reaction of one molecule of re-

agent with one vital group. This does not, however, exclude the existence of more than one vital sulfhydryl group on the enzyme molecule.

Reaction with each of the two reagents results in parallel and equal inhibition of the Na^+-stimulated ATPase activity, the phosphorylation by ATP and the (Na^++K^+)-ATPase activity (for *N*-ethylmaleimide see ref. 8, Fig. 6 and Table II). Binding of ATP to the enzyme protects against inactivation by both reagents (for *N*-ethylmaleimide see ref. 8, Table I). The ATP concentration for 50% protection against complete inhibition by 5,5′-dithiobis-(2-nitrobenzoic acid) amounts to 20 μM, in good agreement with the value (15 μM) calculated from Fig. 2 of ref. 7. The dissociation constant for ATP towards the site protected by it against 5,5′-dithiobis-(2-nitrobenzoic acid) calculated according to the method of Carter et al. [19] from the data in Fig. 5, is 5 μM. The corresponding dissociation constant for ATP for protection against *N*-ethylmaleimide inhibition, calculated from the data in Fig. 2, ref. 8, amounts to 20 μM. These values approach the K_D value (0.13—0.22 μM) for the binding of ATP [20,21], suggesting that ATP protects in both cases through binding to the high affinity site on the enzyme. This may occur either by screening off a sulfhydryl group inside or adjacent to the ATP binding center or by inducing a conformational change of the enzyme leading to shielding of a vital sulfhydryl group elsewhere.

There is some evidence favouring the former alternative, viz. the localization of a vital sulfhydryl group in the active center. First, there is qualitative agreement between the effects of 5,5′-dithiobis-(2-nitrobenzoic acid) and of the ATP binding site-directed sulfhydryl reagent *S*-2,4-dinitrophenyl 6-mercaptopurine riboside [7] on the activities of (Na^++K^+)-activated ATPase and K^+-stimulated 4-nitrophenylphosphatase. Secondly, prior treatment with 5,5′-dithiobis-(2-nitrobenzoic acid) has an effect on the reaction rate of 2,3-butanedione with an arginyl residue situated in the ATP binding center [22].

Comparison of the effects of 5,5′-dithiobis-(2-nitrobenzoic acid) with those of *N*-ethylmaleimide also shows a number of differences. First, after reaction with *N*-ethylmaleimide the K^+-stimulated 4-nitrophenylphosphatase activity is inhibited to the same extent as the (Na^++K^+)-ATPase activity, whereas after reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) the phosphatase activity is inhibited significantly less. Secondly, the protecting effect of ATP on the inactivation of the phosphatase activity occurs in the case of *N*-ethylmaleimide only under non-phosphorylating conditions, whereas in the case of 5,5′-dithiobis-(2-nitrobenzoic acid) protection occurs both under phosphorylating and under non-phosphorylating conditions. Thirdly, 4-nitrophenylphosphate antagonizes the inhibition of the (Na^++K^+)-ATPase activity by *N*-ethylmaleimide, but not that by 5,5′-dithiobis-(2-nitrobenzoic acid). Fourthly, 4-nitrophenyl phosphate antagonizes the inhibition of the phosphatase activity by *N*-ethylmaleimide under non-phosphorylating conditions only, but that by 5,5′-dithiobis-(2-nitrobenzoic acid) under both phosphorylating and non-phosphorylating conditions. Finally, there is a difference in the number of sulfhydryl groups modified by each reagent in the absence of detergent. 5,5′-Dithiobis-(2-nitrobenzoic acid) reacts with only 12 sulfhydryl groups per enzyme molecule whereas *N*-ethylmaleimide can modify 26 out of a total of 36 sulfhydryl groups.

These differences in the effects of both reagents on the partial and overall

activities strongly suggest that different vital groups are modified. This is confirmed by the experiment in which the enzyme is made to react with *N*-ethylmaleimide after treatment with 5,5′-dithiobis-(2-nitrobenzoic acid). The inhibition by 5,5′-dithiobis-(2-nitrobenzoic acid) alone can be reversed by subsequent treatment with dithioerythritol. However, after treatment of the 5,5′-dithiobis-(2-nitrobenzoic acid)-inhibited enzyme with *N*-ethylmaleimide, no restoration of the ATPase activity is observed upon incubation with dithioerythritol. This proves that *N*-ethylmaleimide has reacted with a vital group, which has not been blocked by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid). The fact that *N*-ethylmaleimide modifies a larger number of sulfhydryl groups than the other reagent suggests that this vital group is also a sulfhydryl group. This is further supported by our finding that inhibition of $(Na^+ + K^+)$-ATPase activity through modification of amino groups by means of alkylimidate compounds is not antagonized by ATP (J.J.H.H.M. de Pont et al., to be published).

The reaction of *N*-ethylmaleimide with the enzyme after prior treatment with 5,5′-dithiobis-(2-nitrobenzoic acid) can be partially prevented by ATP. From the degree of protection obtained by 10 mM ATP it appears that in this case a low affinity ATP binding site is involved. This may be due either to a change in affinity of the former high affinity site by prior reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) or else to the presence of a low affinity ATP binding site, which is not modified by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid).

The total number of sulfhydryl groups on the enzyme, determined with 5,5′-dithiobis-(2-nitrobenzoic acid) in the presence of sodium dodecyl sulphate (36 ± 2) is within the range of the numbers (51, 28 and 24, respectively) calculated from the data reported by Kyte [23], Hopkins et al. [24] and Perrone et al. [25]. Combination of the results described in this and the previous paper [8] leads to the following classification of these 36 sulfhydryl groups:

Class A, containing 12 sulfhydryl groups reacting with both *N*-ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid), inclusive one that is vital for the enzyme activity.

Class B, containing (at least) 14 sulfhydryl groups reacting only with *N*-ethylmaleimide, one of which is vital.

Class C, containing (at most) 10 sulfhydryl groups that react with neither 5,5′-dithiobis-(2-nitrobenzoic acid) nor *N*-ethylmaleimide in the absence of detergent. About the presence of a vital sulfhydryl group in this class nothing can be said.

From the octanol/water partition coefficients of *N*-ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid) it appears that the class A sulfhydryl groups must be located on the hydrophilic surface of the enzyme, whereas the class B sulfhydryl groups seem to be present in a more hydrophobic environment. The class C sulfhydryl groups must be located in very inaccessible parts of the molecule, which are only exposed upon denaturation by sodium dodecyl sulphate.

Thus two different types of vital sulfhydryl groups are present in $(Na^+ + K^+)$-ATPase, one hydrophilic type, reacting with both reagents and probably located in the ATP binding site (class A), and one more hydrophobic type, reacting with *N*-ethylmaleimide only (class B) and probably not located in an

active center (see ref. 8). There appear to be two different class A vital groups, one located in the high affinity ATP binding site, the other involved in the phosphatase activity. This is demonstrated by the observation of inhibition of the $(Na^+ + K^+)$-ATPase activity by 5,5'-dithiobis-(2-nitrobenzoic acid) under conditions where inhibition of the phosphatase activity is antagonized (presence of ATP under phosphorylating conditions or of 4-nitrophenyl phosphate under both phosphorylating and non-phosphorylating conditions).

Note added in proof (Received September 13th, 1977)

Recent experiments with ^{3}H-labeled *N*-ethylmaleimide followed by SDS gel electrophoresis indicate that virtually all reactive SH groups in the native enzyme are located in the 100 000 M_r subunits(s) (Schoot et al., to be published).

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