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STUDIES ON ($K^+ + H^+$)-ATPase

II. ROLE OF SULFHYDRYL GROUPS IN ITS REACTION MECHANISM

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Summary

1. Preincubation of a microsomal membrane preparation from pig gastric mucosa with 5,5'-dithiobis(2-nitrobenzoic acid) inhibits the ($K^+ + H^+$)-ATPase and K^+-stimulated 4-nitrophenylphosphatase activities of the preparation.

2. The activating monovalent cation, K^+, in the presence of CDTA, increases the rate of inactivation of ($K^+ + H^+$)-ATPase by 5,5'-dithiobis(2-nitrobenzoic acid), indicating a conformational change in the presence of K^+.

3. In the absence of added Mg^{2+} or in the presence of CDTA, the semi-logarithmic plot of ATPase activity vs. reaction time is linear. Upon addition of Mg^{2+}, a fast and a slow phase become discernible.

4. The nucleotides ATP, ADP and dATP in 0.5 mM concentration strongly protect the enzyme against inactivation by the modifying agent in the presence of CDTA. The ATP analogues AMPPNP and AMPPCP exert moderate protection, whilst other nucleotides like GTP, ITP and CTP show only minor protection.

5. Analysis of the protective effect of ATP indicates that in the presence of 2 mM CDTA, 5,5'-dithiobis(2-nitrobenzoic acid) and ATP do not compete for the same site, suggesting that the essential sulfhydryl groups are not located in the ATP binding site.

6. The slow inactivation phase, which occurs in the presence of Mg^{2+}, is completely blocked by ATP, and its non-phosphorylating analogue AMPPNP now appears to be equally potent.

Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; AMPPCP, adenyl(β,γ-methylene)-diphosphate; AMPPNP, adenylyl imidodiphosphate.

7. Titration with 5,5′-dithiobis(2-nitrobenzoic acid) after solubilization in sodium dodecyl sulfate reveals the presence of 71 μmol reactive sulfhydryl groups per gram protein. Under native conditions, approx. 50% of these are modified in 30 min of incubation with the modifying agent. In the presence of Mg^{2+}, the number of rapidly reacting groups is increased.

8. Incubation with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of Mg^{2+}, but not in its absence, results in a decreased phosphorylation level. On the other hand, treatment in the absence of Mg^{2+}, but not in its presence, decreases the K^+-stimulated dephosphorylation of the enzyme.

9. The results are discussed in terms of a Mg^{2+}-induced conformational change of the gastric ($K^+ + H^+$)-ATPase. It is suggested that sulfhydryl groups, which are modified in the presence of Mg^{2+}, are involved in a step leading to enzyme phosphorylation. Other sulfhydryl groups, which are modified in the absence of Mg^{2+}, seem to be involved in the K^+-stimulated dephosphorylation step.

Introduction

Microsomal membrane preparations, derived from fundic gastric mucosa of several species, contain an ouabain-insensitive, Mg^{2+}-dependent, K^+-activated ATPase activity [1]. The isolated membrane fraction, consisting mostly of closed vesicles, is capable of H^+ transport in the presence of K^+, Mg^{2+} and ATP [2]. This proton transport is accompanied by a K^+ efflux in an apparently electroneutral fashion [3—5], hence the abbreviation ($K^+ + H^+$)-ATPase is used for the enzyme. The ($K^+ + H^+$)-ATPase-containing membranes are largely derived from parietal cells [6]. Recent immunological studies have shown that the enzyme is located in that region of the acid-secreting cell, where the site of acid secretion is postulated [7]. It is, therefore, assumed that the enzyme is involved in gastric acid secretion (see reviews 8—10).

The ($K^+ + H^+$)-ATPase reaction mechanism resembles that of the other known transport ATPases. The reaction scheme includes binding of ATP to the enzyme, followed by Mg^{2+}-activated phosphorylation and K^+-activated dephosphorylation of the enzyme [8—11]. However, still relatively little is known about the molecular structure and the detailed reaction mechanism of the enzyme.

More insight into the reaction mechanism of the enzyme has recently been obtained by studying the effects of modification of the enzyme, e.g. by means of proteolytic enzymes like trypsin [12]. Group-specific modification by chemical reagents can also provide useful information about catalytic properties and conformational states of the enzyme. Our previous studies with the arginine reagent, butanedione, have shown that the enzyme has at least two conformational states, a Mg^{2+}-induced and a K^+-induced state [13]. Incubation with the sulfhydryl reagents, *N*-ethylmaleimide and 5,5′-dithiobis(2-nitrobenzoic acid), leads to inactivation of the valinomycin-stimulated part of the ATPase activity as described by Lee et al. [14]. In an earlier report of the same group, it was postulated that sulfhydryl groups might be involved in the formation of the enzyme-substrate complex and the dephosphorylation step [11].

In the present study, the effects of 5,5′-dithiobis(2-nitrobenzoic acid) on the overall ATPase activity and on some partial reactions of the system have been investigated. Membrane preparations which have lost their vesicular structure (no enhancement of activity in the presence of ionophore) have been used. The results are compared with data, obtained from similar studies on ($Na^+ + K^+$)-ATPase [15,16] and ($Ca^{2+} + Mg^{2+}$)-ATPase [17,18].

Materials and Methods

Isolation of $K^+ + H^+$)-ATPase-containing membranes. Gastric mucosal membranes are obtained as previously described [13]. Briefly, pig gastric mucosal homogenate is centrifuged at 20 000 × *g* for 20 min and the resulting supernatant is centrifuged on a 37% (w/v) sucrose cushion for 30 min at 100 000 × *g*, yielding a microsomal fraction at the interface. After dilution, the interface fraction is distributed by zonal centrifugation on a linear gradient, ranging from 20 to 43% (w/v) sucrose in 25 mM Tris-HCl (pH 7.4). After dilution, the fraction with a density of 1.11—1.13 is collected by centrifuging for 1 h at 100 000 × g_{av}. The resulting pellet is routinely resuspended in 0.25 M sucrose to a protein concentration of 1 mg/ml and is then added dropwise to liquid nitrogen. The resulting beads are stored at —20°C.

Treatment with 5,5′-dithiobis(2-nitrobenzoic acid). The reaction is performed in a medium containing 25 mM imidazole hydrochloride (pH 7.3), 0.125 M sucrose and 0.5 mg protein/ml. pH 7.3 has been chosen to allow simultaneous determination of sulfhydryl groups, since at this pH, both the hydroxy-promoted hydrolysis of 5,5′-dithiobis(2-nitrobenzoic acid) is minimal and the product 5-thio-2-nitrobenzoic acid is for 99.8% in the intensely colored conjugate base form [19]. When additives are present (concentrations indicated in the text), the gastric membranes are preincubated at 37°C for 10 min before addition of the modifying agent. A solution of 5,5′-dithiobis(2-nitrobenzoic acid) in the same buffer (preincubated at 37°C) is then added and the mixture is incubated at 37°C. Aliquots of 200 μl are removed at certain times. Separation of the protein from the reaction mixture is achieved by gel filtration at room temperature over a Sephadex G-25 coarse column (0.5 × 10 cm), which has previously been equilibrated in 25 mM imidazole hydrochloride (pH 7.3). The same buffer is used for elution. The first eluate after addition of 200 μl elution buffer is discarded, whilst the second eluate obtained after adding 700 μl elution buffer is used for enzyme assays and protein determination. Control samples without 5,5′-dithiobis(2-nitrobenzoic acid), treated in the same way, are always included.

Determination of sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid). This is carried out by reading the 412 nm absorbance of 5-thio-2-nitrobenzoic acid, which is released during reaction of the enzyme with the parent reagent. Incubation of gastric membranes with 5,5′-dithiobis(2-nitrobenzoic acid) is performed as described above for the inactivation studies, except that now the incubation mixture is continuously gassed with nitrogen. After suitable times, the protein is removed by pressing 500 μl samples through a Millipore filter (Gs 0.22 μm) in a Swinnex filter system (Millipore, Molsheim, France). A 380-μl volume of filtrate is added to 20 μl 80 mM CDTA and is stored under nitrogen

until the absorbance at 412 nm can be read (within 1 h) using a molar absorption coefficient of 14 150 [19]. This procedure gives rather reproducible results, probably due to the fact that in the presence of CDTA the metal ion-catalyzed oxidation of 5-thio-2-nitrobenzoic acid is prevented [20].

When Mg^{2+} is present during incubation, the filters are occluded more often than in its absence, suggesting vesicle aggregation or formation of larger protein complexes. This may be due to extensive cross-linking of ATPase molecules by the modification reagent in this condition. Indications for cross-linking are also described after treatment of Ca^{2+}-ATPase with 5,5'-dithiobis(2-nitrobenzoic acid) [17].

Phosphorylation of gastric membranes by [γ-^{32}P]ATP. Phosphorylation is carried out in the medium, which after addition of an equal volume of a membrane suspension, contains 50 mM imidazole hydrochloride (pH 7.0), 1 mM $MgCl_2$ and 20 μM [γ-^{32}P]ATP. The reaction is started by adding 50 μl enzyme preparation (25—50 μg protein) to the phosphorylation medium. After 10 s at room temperature, the reaction is stopped by addition of 2 ml 5% (w/v) trichloroacetic acid containing 0.1 M H_3PO_4. By determining the time dependence of the phosphorylation reaction at room temperature, we find that the membranes reach the maximal phosphorylation level in 10 s. The precipitated membranes are collected by filtration on a 1.2 μm pore width Selectron filter (Schleicher & Schüll, Dassel, F.R.G.), which is then washed three times with stopping solution (total volume 18 ml). Membrane-bound ^{32}P is determined by liquid-scintillation counting. Blanks are prepared by mixing stopping solution with [γ-^{32}P]ATP prior to enzyme addition.

When the effect of incubation with 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) on the phosphorylation level is investigated, 50-μl aliquots of the incubation mixture (37°C) containing 25 μg protein are directly added to the phosphorylation medium (0°C). Consequently, 50 μM 5,5'-dithiobis(2-nitrobenzoic acid) and 0.063 M sucrose are present during phosphorylation at 18°C after mixing. If Mg^{2+} is present in the inactivation mixture, the composition of the phosphorylation medium is changed in such a way that during phosphorylation the Mg^{2+} concentration amounts to 1 mM.

Dephosphorylation of ^{32}P-labelled gastric membranes. Dephosphorylation is measured by adding 100 μl of a non-radioactive ATP solution (2 mM) to gastric membranes phosphorylated for 20 s, as described above. After suitable times at room temperature, dephosphorylation is stopped by addition of the stopping solution. The precipitated membranes are treated as described in the previous section.

K^+-stimulated dephosphorylation is studied by including 10 mM K^+ in the ATP solution.

Enzyme assays and protein determination. ($K^+ + H^+$)-ATPase and K^+-stimulated 4-nitrophenylphosphatase are assayed by incubating enzyme preparations for suitable times in the presence of 30 mM imidazole hydrochloride (pH 7.0), 5 mM Na_2ATP (or 5 mM *p*-nitrophenylphosphate), 5 mM $MgCl_2$, 0.1 mM ouabain and 20 mM KCl or choline chloride, as previously described [13]. Protein is determined according to Lowry et al. [21].

Materials. 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 specific radioactiv-

ity) from The Radiochemical Center (Amersham, U.K.). All nucleotides are purchased from Boehringer (Mannheim, F.R.G.), ouabain, Tris, 4-nitrophenylphosphate (disodium salt) 1,4-dithioerythritol, CDTA and imidazole from Merck (Darmstadt, F.R.G.). All other chemicals are of reagent grade.

Results

Modification of gastric ($K^+ + H^+$)-ATPase by 5,5′-dithiobis(2-nitrobenzoic acid)

The reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with gastric ($K^+ + H^+$)-ATPase results in a rapid loss of enzyme activity (Fig. 1). The presence of 1 mM 1,4-dithioerythritol prevents inactivation, while its addition after 50% inactivation of the enzyme leads to restoration of the ($K^+ + H^+$)-ATPase activity.

In the absence of any ligand and in the presence of 2 mM CDTA, the inactivation by 5,5′-dithiobis-(2-nitrobenzoic acid) shows pseudo-first-order kinetics, while ATP protects against inactivation (not shown). In the presence of 2 mM CDTA and 0.1 mM sulfhydryl reagent, the pseudo-first-order reaction constant is $-0.044\ min^{-1}$ (S.E. = 0.003, n = 19; range -0.033 to -0.072.) The K^+-stimulated 4-nitrophenylphosphatase activity is inhibited to the same extent as the ATPase activity (not shown).

A plot of the pseudo-first-order rate constant of inactivation versus the concentration of 5,5′-dithiobis(2-nitrobenzoic acid) is non-linear and exhibits saturation kinetics (not shown). This can be due to a reversible complex formation of the modifying agent with the enzyme prior to the covalent modification reaction yielding the inactive enzyme [22]. This may also explain why we have previously found a plateau for the residual activity when gastric membranes are incubated with different concentrations of 5,5′-dithiobis(2-nitrobenzoic acid) for 60 min [23]. Moreover, at high concentrations (greater than or equal to 1 mM) of the reagent, deviation from pseudo-first-order kinetics occurs, indicating more complex kinetics (not shown).

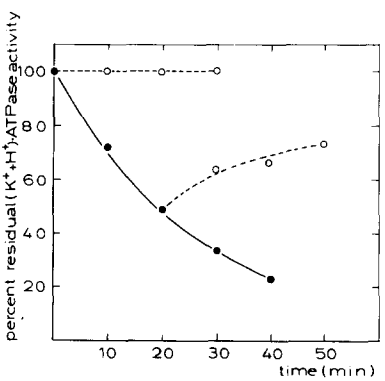

Fig. 1. Reversible inactivation of ($K^+ + H^+$)-ATPase by 5,5′-dithiobis(2-nitrobenzoic acid). Enzyme preparation (0.57 mg protein/ml) is incubated at 37°C during the indicated times with 100 μM reagent, 25 mM imidazole hydrochloride (pH 7.3) and 2 mM CDTA. Restoration of enzyme activity is followed by addition of 1 mM 1,4-dithioerythritol after 20 min incubation with the modifying agent. Enzyme activity is determined as described in the text and is expressed as percent of control activity without 5,5′-dithiobis(2-nitrobenzoic acid)-treatment.

TABLE I

PROTECTIVE EFFECTS OF NUCLEOTIDES ON INACTIVATION OF $(K^+ + H^+)$-ATPase BY 5,5′-DITHIOBIS(2-NITROBENZOIC ACID) IN THE PRESENCE OF CDTA

$(K^+ + H^+)$-ATPase (0.46 mg protein/ml) is preincubated for 10 min at 37°C in a medium containing 25 mM imidazole hydrochloride (pH 7.3), 2 mM CDTA and 0.5 mM of the indicated compounds, after which it is incubated in the same medium, containing in addition 100 μM 5,5′-dithiobis(2-nitrobenzoic acid). After suitable times, the reaction is stopped by gel filtration and the residual enzyme activity is determined. The slope (V_i) of a semilogarithmic plot of the residual activity vs. time of reaction is determined. V_o is the slope in the absence of nucleotides. The S.E. from two experiments is calculated as 0.63X the range, according to the approximation method of Davies and Pearson [27].

Nucleotide present (0.5 mM)	V_i/V_o with S.E. (n = 2)
ATP	0.22 ± 0.01
dATP	0.31 ± 0.01
ADP	0.28 ± 0.02
AMPPNP	0.43 ± 0.02
AMPPCP	0.59 ± 0.01
GTP	0.79 ± 0.03
CTP	0.82 ± 0.07
ITP	0.86 ± 0.02
AMP	0.96 ± 0.01
HPO_4^{2-}	0.95 ± 0.01

The activating monovalent cation K^+ increases the rate of enzyme inactivation by 5,5′-dithiobis(2-nitrobenzoic acid) in the absence of Mg^{2+} (CDTA added), following pseudo-first-order kinetics. The pseudo-first-order reaction constant in the presence of 1 mM K^+, 2 mM CDTA and 0.1 mM reagent is -0.055 min^{-1} (S.E. = 0.002; n = 4), which is significantly different from the mean pseudo-first-order reaction constant in the paired control experiments (-0.044 min^{-1}; S.E. = 0.002). From experiments in which the effects of varying the KCl concentration on the inactivation rate was studied, dissociation constants for the enzyme-cation complex have been calculated by means of the

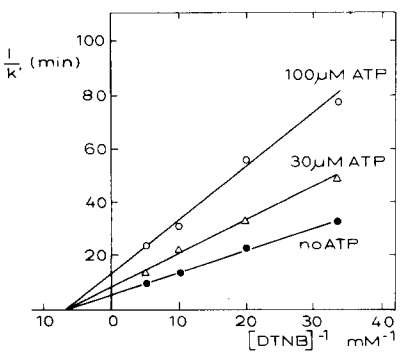

Fig. 2. Inactivation of $(K^+ + H^+)$-ATPase by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of ATP. Enzyme preparation (0.5 mg protein/ml) is incubated at 37°C with different concentrations of the reagent in 25 mM imidazole hydrochloride (pH 7.3), 2 mM CDTA and 0, 30 or 100 μM ATP. At appropriate times, the reaction is stopped by gel filtration (see Materials and Methods). Apparent first-order rate constants of inactivation are determined. The graph shows double-reciprocal plots of these constants vs. reagent concentration.Typical experiment out of three.

Scrutton and Utter equation [25]. In the presence of 2 mM CDTA a K_d value of 0.044 ± 0.002 mM (n = 3) is found for K^+, which is somewhat higher than that calculated for the protecting effect of K^+ against butanedione inactivation [13].

In the presence of 100 μM ATP and 2 mM CDTA, K^+ also increase the inactivation rate. By varying the K^+ concentration in this case, a K_d of 0.54 mM for K^+ is found, which is 12-times as high as in the absence of ATP.

Protection by nucleotides against inactivation by 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of CDTA

The effects of various nucleotides (0.5 mM) on the inactivation of gastric ($K^+ + H^+$)-ATPase by 5,5'-dithiobis(2-nitrobenzoic acid) in the absence of Mg^{2+} (CDTA added) are shown in Table I. ATP, dATP and ADP protect most efficiently. The non-phosphorylating ATP analogues AMPPNP and AMPPCP protect moderately, while the presence of GTP, ITP, CTP, AMP and inorganic phosphate has little effect.

We have examined whether 5,5'-dithiobis(2-nitrobenzoic acid) mimics the substrate ATP at the substrate binding site. The dissociation constant for the enzyme-reagent complex is independent of the ATP concentration (Fig. 2), indicating that the reagent and ATP are not competing for the same site.

Effect of Mg^{2+} on the inactivation of ($K^+ + H^+$)-ATPase by 5,5'-dithiobis(2-nitrobenzoic aicd)

In the presence of Mg^{2+}, a fast and a slow phase become discernible (Fig. 3). This effect is also observed at a reagent concentration of 30 μM instead of 100 μM. As mentioned above, in the absence of added Mg^{2+} (with or without

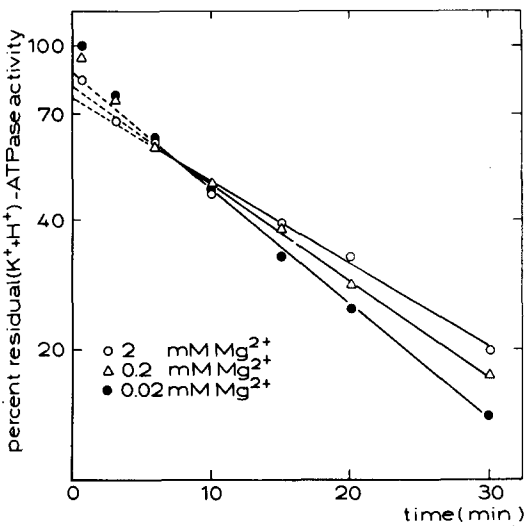

Fig. 3. Effect of Mg^{2+} on inactivation of ($K^+ + H^+$)-ATPase by 5,5'-dithiobis(2-nitrobenzoic acid). Enzyme preparation (0.5 mg protein/ml) is incubated at 37°C during the indicated times with 100 μM reagent in 25 mM imidazole hydrochloride (pH 7.3), containing 0.02, 0.2 or 2 mM $MgCl_2$. Enzyme activity is assayed as described under Materials and Methods. Typical experiment out of four.

CDTA), linear semilogarithmic plots are obtained at reagent concentrations below 1 mM.

From Fig. 3 it can be seen that increasing the Mg^{2+} concentration has two effects: (1) the extent of inhibition during the initial rapid phase increases. Depending on the preparation, the maximal extent obtained by extrapolation of the slow phase amounts to 22—30% inhibition at 0.5 mM Mg^{2+} or more. (2) the activation rate in the slow phase is lowered. In this phase and in the presence of 2 mM Mg^{2+}, it is $-0.034\ min^{-1}$ (S.E. = 0.004, $n = 4$).

The inactivation in the slow phase can be completely abolished by ATP (Fig. 4A and B). It is obvious that in the presence of 0.5 mM Mg^{2+}, less ATP is needed for complete protection than in the presence of 20 μM Mg^{2+}. This would be understandable, if the Mg-ATP complex is a better protector than free ATP. The inactivation of the K^+-stimulated 4-nitrophenylphosphatase activity by 5,5′-dithiobis(2-nitrobenzoic acid) (Fig. 4C and D) parallels that of the (K^+ + H^+)-ATPase activity.

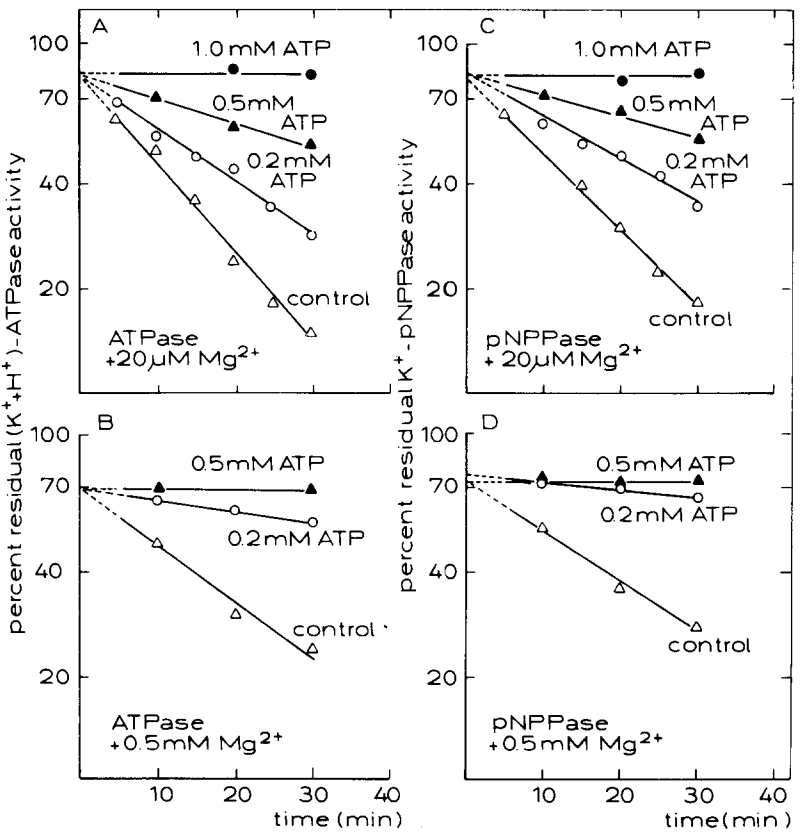

Fig. 4. Effect of ATP on inactivation of (K^+ + H^+)-ATPase and K^+-activated 4-nitrophenylphosphatase by 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of Mg^{2+}. Enzyme preparation (0.5 mg protein/ml) is incubated at 37°C during the indicated times with 100 μM reagent in 25 mM imidazole hydrochloride (pH 7.3), containing 20 μM $MgCl_2$ (A and C) or 0.5 mM $MgCl_2$ (B and D) and 0, 0.2, 0.5 or 1.0 mM ATP. (K^+ + H^+)-ATPase (A and B) and K^+-activated 4-nitrophenylphosphatase (C and D) activitites are assayed as described under Materials and Methods.

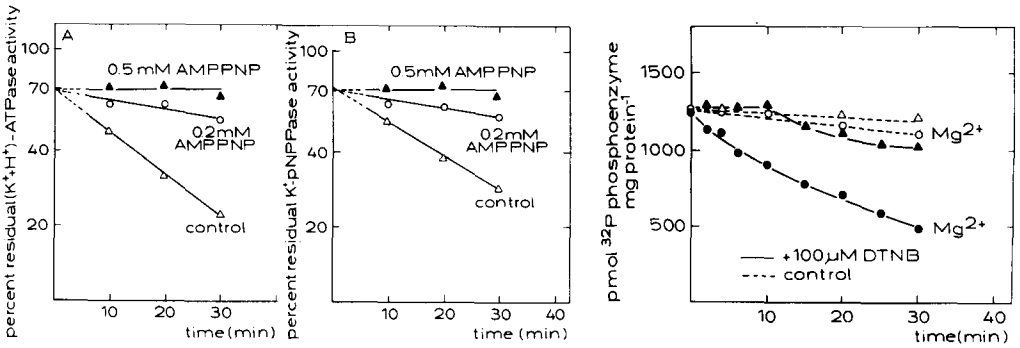

Fig. 5. Effect of AMPPNP on inactivation of $(K^+ + H^+)$-ATPase and K^+-activated 4-nitrophenylphosphatase by 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of 0.5 mM Mg^{2+}. Enzyme preparation (0.5 mg protein/ml) is incubated at 37°C during the indicated times with 100 µM reagent in 25 mM imidazole hydrochloride (pH 7.3), 0.5 mM $MgCl_2$ and 0, 0.2 or 0.5 mM AMPPNP. $(K^+ + H^+)$-ATPase (A) and K^+-activated 4-nitrophenylphosphatase (B) activities are determined as described under Materials and Methods.

Fig. 6. Phosphorylation levels of $(K^+ + H^+)$-ATPase after incubation with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence or absence of Mg^{2+}. Enzyme preparation (0.62 mg protein/ml) is incubated at 37°C with 100 µM reagent in 25 mM imidazole hydrochloride (pH 7.3) with or without 2 mM Mg^{2+}. At the indicated times, samples of the incubation mixtures are phosphorylated for 10 s in the presence of 50 mM imidazole hydrochloride (pH 7.0), 1 mM $MgCl_2$ and 20 µM [^{32}P]ATP, as described under Materials and Methods. Controls without the modifying reagent are treated in the same way. Typical experiment out of four.

Similar experiments with AMPPNP exhibit the same picture (Fig. 5). The protection by AMPPNP in the presence of Mg^{2+} equals that by ATP, while in the presence of CDTA, we find less protection by AMPPNP than by ATP (Table I).

Titration of sulfhydryl groups of $(K^+ + H^+)$-ATPase in the presence of CDTA or Mg^{2+}

The effects of Mg^{2+} on enzyme inactivation by 5,5′-dithiobis(2-nitrobenzoic acid) have led us to investigate the number of sulfhydryl groups which can be modified in the presence or absence of Mg^{2+}. During the first 30 min, there is a faster increase of the 412 nm absorbance by 5-thio-2-nitrobenzoic acid in the presence of Mg^{2+} than in the presence of 2 mM CDTA (Table II). The total number of sulfhydryl groups of 71 ± 2 µmol/g protein measured in the presence of 1% (w/v) sodium dodecyl sulfate is in fair agreement with the number of approx. 80 µmol sulfhydryl groups/g protein reported by Lee et al. [14].

Effects of sulfhydryl modification on the levels of phosphorylation and K^+-dependent dephosphorylation

The effects of inactivation of $(K^+ + H^+)$-ATPase with 5,5′-dithiobis(2-nitrobenzoic acid) in the absence or presence of Mg^{2+} on the formation of ^{32}P-labeled phosphoenzyme was studied (Fig. 6). Incubation with the reagent in the absence of Mg^{2+} does not affect the phosphorylation level during the first few minutes of incubation. In the presence of Mg^{2+}, however, an immediate decrease of the phosphorylation level is observed. The pseudo-first-order rate

TABLE II

DETERMINATION OF THE NUMBER OF REACTIVE SULPHYDRYL GROUPS IN $(K^+ + H^+)$-ATPase IN THE PRESENCE OF CDTA OR Mg^{2+}

$(K^+ + H^+)$-ATPase preparation (0.5 mg protein/ml) is incubated at 37°C with 100 μM 5,5′-dithiobis(2-nitrobenzoic acid) in 25 mM imidazole hydrochloride (pH 7.3) in the presence of 2 mM CDTA or 2 mM $MgCl_2$, as described under Materials and Methods. After 15 or 30 min of incubation, the number of modified sulfhydryl groups is determined spectrophotometrically. The total number of sulfhydryl groups is measured by adding 1% (w/v) sodium dodecyl sulfate to the incubation mixture. The values are expressed as μmol SH/g protein with S.E. and in parentheses are given the number of experiments. SDS, sodium dodecyl sulfate.

Additives	Incubation time	
	15 min	30 min
2 mM CDTA	18 ± 1 (5)	30 ± 3 (6)
2 mM Mg^{2+}	22 ± 2 (6)	40 ± 2 (4)
1% SDS	—	71 ± 2 (11)

constant of this effect (−0.033 min^{-1}; S.E. = 0.007, n = 2) equals the rate constant for the slow phase of inactivation in the presence of 2 mM Mg^{2+} (−0.034 min^{-1}; S.E. = 0.004, n = 4). This suggests that the inactivation of enzyme activity observed in the slow phase in Fig. 3 is due to modification of vital sulfhydryl groups, which are involved in a step leading to phosphorylation of the enzyme (ATP binding and/or phosphoryl transfer). This has also been concluded for the inactivation of $(Ca^{2+} + Mg^{2+})$-activated ATPase by this sulfhydryl reagent [17].

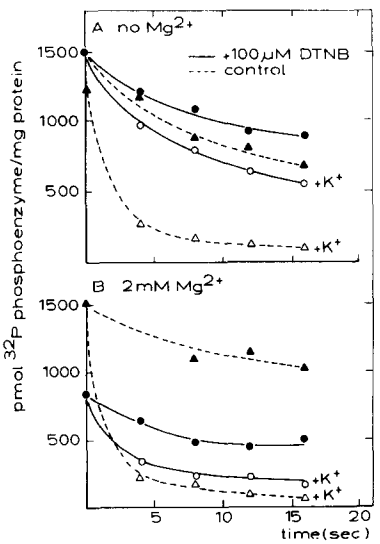

Fig. 7. Effect of Mg^{2+} on dephosphorylation of $(K^+ + H^+)$-ATPase after treatment with 5,5′-dithiobis(2-nitrobenzoic acid). Enzyme preparation (0.36 mg protein/ml) is incubated at 37°C for 30 min with 100 μM reagent in 25 mM imidazole hydrochloride (pH 7.3) in the absence (A) or presence (B) of 2 mM $MgCl_2$. After 30 min, the incubated enzyme is phosphorylated for 20 s in the presence of 50 mM imidazole hydrochloride (pH 7.0), 1 mM $MgCl_2$ and 20 μM [^{32}P]ATP. Dephosphorylation is measured during the indicated times by adding carrier-free ATP (final concentration 1 mM) with or without 5 mM K^+ present, as described under Materials and Methods. Control without modifying reagent are treated in the same way.

Investigation of the dephosphorylation of the enzyme after incubation with 5,5′-dithiobis(2-nitrobenzoic acid) in the absence or presence of Mg^{2+} reveals another interesting point. When the enzyme is modified in the presence of Mg^{2+}, the K^+-dependent dephosphorylation of the ^{32}P-labeled phosphoenzyme is not affected (Fig. 7B). This is so because in the presence of 1 mM ATP and 5 mM K^+ nearly the same level of phosphorylation as in a control preparation (not treated with the reagent) is reached. When the enzyme is inactivated at shorter incubation times (2 and 10 min; not shown), the same low level of ^{32}P-labeled phosphoenzyme is reached after 16 s.

On the other hand, incubation with the reagent in the absence of Mg^{2+} results in a decreased sensitivity of the dephosphorylation for K^+ (Fig. 7A). This indicates that in the absence of Mg^{2+} vital sulfhydryl groups are modified, which are involved in K^+-sensitive dephosphorylation of the ^{32}P-labeled phosphoenzyme.

Discussion

Modification of the sylfhydryl groups of many enzymes leads to their inactivation. In most cases, it is difficult to distinguish between sulfhydryl groups present in an active site and peripheral sulfhydryl groups which have a function in the maintenance of enzyme conformation through ionic or hydrogen-binding interactions.

The experiments reported in this paper, clearly indicate that a $(K^+ + H^+)$-ATPase preparation from porcine gastric mucosa is inhibited by treatment with 5,5′-dithiobis(2-nitrobenzoic acid). The inhibition reaction is dependent on the presence in the reaction medium of ligands like K^+, Mg^{2+} or nucleotides. The inhibition by the modifying agent can be reversed by subsequent treatment with dithioerythritol.

In the presence of CDTA, the inactivation by 5,5′-dithiobis(2-nitrobenzoic acid) shows pseudo-first-order kinetics and can be fitted by linear semilogarithmic plots.This suggests that all sulfhydryl groups the modification of which leads to inactivation of the enzyme, react at the same rate.

We cannot exclude that CDTA per se has an effect on the $(K^+ + H^+)$-ATPase, as is found in the AMPPNP binding studies [26]. Such an effect has not been observed in these studies. The semilogarithmic inactivation curve and the pseudo-first-order inactivation constant are not affected by the presence of CDTA.

The effects of K^+ on the inactivation of gastric $(K^+ + H^+)$-ATPase by 5,5′-dithiobis(2-nitrobenzoic acid) must reflect ion-induced conformational changes. K^+ increases the rate of inactivation, an effect which is opposite to its effect on the inactivation by butanedione in the absence of ATP, where it protects the essential arginine residue. This may be an indication that the essential sulfhydryl groups are not located in the substrate binding site. In the presence of ATP, K^+ also increases the inactivation rate, but the K^+ concentration required for a maximal effect is increased (K_d = 0.044 mM in the absence and K_d = 0.54 mM in the presence of ATP). This suggests that ATP and K^+ have antagonistic effects when they bind to the enzyme, which is also suggested by the butanedione inactivation studies. Direct AMPPNP-binding studies with gastric mem-

branes show that K^+ do indeed decrease the binding of this nucleotide [26]. In these binding experiments, the same order of effectiveness ($Tl^+ > K^+ > Rb^+$) is found as described for their stimulation of the dephosphorylation of the phosphoenzyme [11]. This suggests that the cation-induced conformational change is related to the dephosphorylation process.

The non-competitive nature of the protection by ATP against modification by 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of CDTA, indicates that the reagent is not bound at the nucleotide binding site. This suggests that the protective effect is due to a conformational change, which coincides with nucleotide binding and which leads to decreased exposure of the sulfhydryl groups. ($Ca^{2+} + Mg^{2+}$)-activated ATPase from sarcoplasmic reticulum shows a similar protective effect of ATP on the modification by this reagent. This effect is ascribed to the slowing of the modification reaction and not to the masking of a particular sulfhydryl group [17]. In both enzymes, the modification rate may be influenced by a conformational change as well as by the binding of ligands. The possibility that ATP binds at a site in the vicinity of the reactive sulfhydryl groups can, therefore, not be excluded.

The relative protective effects of other nucleotides on sulfhydryl modification resemble their effects on the inactivation by butanedione [13], except that here the effect of ATP is much less specific. The latter finding is in line with the assumption that the essential arginine residue modified by butanedione is located in the ATP binding site, while the sulfhydryl group modified in the presence of CDTA is located elsewhere.

Modification of sulfhydryl groups without addition of Mg^{2+} or in the presence of CDTA does not affect the phosphorylation level. This is different from the observations for sulfhydryl group modification of ($Na^+ + K^+$)-ATPase [15,16]. However, the activation by K^+ of the dephosphorylation of the ($K^+ + H^+$)-ATPase is greatly decreased. This suggests that the modified sulfhydryl group is involved in K^+-stimulated dephosphorylation, as has also been concluded by Ray and Forte [11] from their experiments with *N*-ethylmaleimide.

The effects of the presence of Mg^{2+} during incubation with 5,5′-dithiobis(2-nitrobenzoic acid) are remarkable. Fast and slow phases of enzyme inactivation appear and the extent of inhibition of ($K^+ + H^+$)-ATPase activity during the initial rapid phase increases with increasing Mg^{2+} concentration. It should be noted that there is a large amount of rather tightly bound endogenous Mg^{2+} present in the isolated membrane preparation [26]. Nevertheless, effects of added Mg^{2+} are observed at concentrations as low as 20 μM. Similar observations have been made for the effect of increasing Ca^{2+} concentrations on the inactivation of ($Ca^{2+} + Mg^{2+}$)-stimulated ATPase from sarcoplasmic reticulum by *N*-ethylmaleimide [18], which the authors described to binding of Ca^{2+} at high-affinity sites on the ($Ca^{2+} + Mg^{2+}$)-ATPase complex. At higher Ca^{2+} concentrations, no effect on the slow phase was observed. For the ($K^+ + H^+$)-ATPase complex, no clearly distinguishable Mg^{2+} sites have been reported so far, although our own studies reveal that Mg^{2+} affects AMPPNP binding in two ways [26]: at low concentrations Mg^{2+} seems to lower the number of binding sites, while Mg^{2+} in higher concentrations competitively inhibits AMPPNP binding. The effects of Mg^{2+} on the maximal extent of inhibition during the initial rapid inactivation phase, described in this paper, occur at the same low

Mg^{2+} concentrations (less than 0.5 mM) as the lowering of the number of binding sites for AMPPNP.

The effects of Mg^{2+} on the slow inactivation phase are probably related to the low-affinity Mg^{2+} effect, because the decrease of the inactivation rate is seen at Mg^{2+} concentrations above 0.5 mM. Moreover, the sulfhydryl groups modified during the slow phase seem to be involved in a step leading to phosphorylation.

The presence of ATP and AMPPNP results in complete protection during the slow inactivation phase. This may be due to total shielding of the essential sulfhydryl groups involved in this process. The nucleotide concentration necessary for complete protection in the presence of Mg^{2+} is smaller than for the protection by these nucleotides in the presence of CDTA. This would suggest that the sulfhydryl groups, modified in the presence of Mg^{2+}, are more directly related to ATP binding. On the other hand, the equal protective effects of ATP and AMPPNP in the presence of Mg^{2+} would indicate that the sulfhydryl group is not located in the ATP binding site, since in that case more specificity of the protective effect would be expected. In any case, there appear to be at least three functionally important sulfhydryl groups, whose modification causes inhibition of different steps of the $(K^+ + H^+)$-ATPase reaction: (1) a sulfhydryl group, modified in the presence of CDTA, which is involved in K^+-activated dephosphorylation, (2) a sulfhydryl group, slowly modified in the presence of Mg^{2+}, which is involved in a step leading to phosphorylation and (3) a sulfhydryl group which is rapidly modified, leading to a small decrease of activity and which may be related to the high-affinity effect or Mg^{2+} on AMPPNP binding.

At the moment, it is not known how specific the effects of Mg^{2+} are and it may well be that other divalent cations can mimic some or all of the Mg^{2+} effects.

In conclusion, our observations appear to reflect a conformational change of the enzyme, which is controlled by Mg^{2+} binding. One possible explanation would be that Mg^{2+} influences the interaction between subunits of the ATPase complex, leading to different reactivity of sulfhydryl groups. A special Mg^{2+}-induced conformation of the enzyme is also indicated by the different numbers of sulfhydryl groups, which are modified under native conditions in the presence of either Mg^{2+} or CDTA. Thus, Mg^{2+} seems to play a crucial role in the $(K^+ + H^+)$-ATPase enzyme complex from gastric mucosa. Since Mg^{2+} is abundantly present in the cell, the Mg^{2+}-induced conformation would seem to be the most prevalent state of the enzyme.

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