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

I. ESSENTIAL ARGININE RESIDUE IN ITS SUBSTRATE BINDING CENTER

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Summary

1. A membrane vesicle fraction containing a high ($K^+ + H^+$)-ATPase activity was isolated from porcine gastric mucosa. The enzyme has a pH optimum of 7.0 and is stimulated by Tl^+, K^+, Rb^+ and NH_4^+ with K_A values of 0.13, 2.7, 7.6 and 26 mM, respectively, at this pH.

2. Incubation of the isolated membrane fraction with butanedione leads to inactivation of the ($K^+ + H^+$)-ATPase activity. The pH-dependence of the inactivation and the reversibility of the reaction, observed after removal of excess butanedione and borate, indicate that modification of arginine is involved.

3. The inactivation of ($K^+ + H^+$)-ATPase activity by butanedione is time-dependent and follows second-order kinetics. From the dependence of the inactivation rate on the reagent concentration it appears that a single arginine residue is involved in the inactivation of the ($K^+ + H^+$)-ATPase activity.

4. ATP, deoxy-ATP, ADP and adenylyl imidodiphosphate (AMPPNP), but not CTP, GTP and ITP which are poor substrates, protect the enzyme against butanedione inactivation, suggesting that the essential arginine residue is located in the ATP binding centre.

5. In the presence of Mg^{2+} the butanedione inactivation is increased, and the protection by ATP, deoxy-ATP and ADP (but not that by AMPPNP) is less pronounced. This suggests that Mg^{2+} induces a conformational change in the enzyme, exposing the arginine group and coinciding with phosphorylation and subsequent release of ADP from its binding site.

Abbreviations: CDTA, cyclohexylene-(1,2)-dinitrotetraacetic acid; AMPPNP, adenylyl imidodiphosphate.

6. Activating monovalent cations protect against butanedione inactivation in the absence of ATP, and do this in the same order of effectiveness ($Tl^+ > K^+ > Rb^+ > NH_4^+$) as for their activation of the ATPase activity. This suggests that these cations induce another conformational state of the enzyme, in which the arginine group is less exposed.

7. In the presence of ATP (Mg^{2+} absent) these cations have a reverse effect: they increase the inactivation by butanedione in the same order of effectiveness. The dissociation constants of Tl^+, K^+ and Rb^+ (0.6, 3.2 and 8.4 mM, respectively) are close to the K_A values for activation by these cations. The cation-induced conformational state, which decreases exposure of the arginine group in the ATP binding centre, thus leads to a lower affinity for ATP. In confirmation of this, we find a 3-fold higher dissociation constant for the enzyme-ATP complex in the presence of 20 mM K^+ than in its absence (216 vs. 74 μM).

Introduction

A K^+-activated ATPase is located in the acid-secreting regions of the gastric mucosa in various species and appears to be involved in gastric acid secretion [1—3]. It seems to transport protons in a neutral exchange for K^+ [4,5], hence the abbreviation ($K^+ + H^+$)-ATPase is used. The enzyme can be isolated in a vesicular membrane fraction. The ATPase activity is activated by monovalent cations in the following order of effectiveness: $Tl^+ > K^+ > Rb^+ > Cs^+$, whilst Na^+, Li^+ and choline$^+$ do not stimulate activity significantly [4,6]. The enzyme contains a 100 000 dalton subunit which is phosphorylated in the presence of ATP and Mg^{2+}. Addition of an activating cation reduces the level of the phosphorylated intermediate [6]. Judging from what is known about its mechanism of action so far, the ($K^+ + H^+$)-ATPase bears a remarkable resemblance to the other known transport ATPases: ($Na^+ + K^+$)-ATPase and ($Ca^{2+} + Mg^{2+}$)-ATPase.

One approach to gain insight into an enzyme mechanism is through chemical modification of particular residues in the enzyme molecule by means of group-specific reagents. Recently, inactivation of gastric ATPase by the sulfhydryl reagents, *N*-ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid), has been reported [7]. In recent years, many cases have been reported where arginine residues exist in the active site of enzymes reacting with anionic substrates or co-factors [8—11], indicating the involvement of the positively-charged side-chain of arginine in substrate or co-factor binding. Such a role for arginine residues has also been established in various ATPases: chloroplast ATPase [12], mitochondrial ATPase [13], ($Ca^{2+} + Mg^{2+}$)-ATPase from sarcoplasmic reticulum [14], and in our laboratory in ($Na^+ + K^+$)-ATPase [15]. In these studies phenylglyoxal, cyclohexanedione or butanedione has been applied as a specific arginine-modifying agent. In this paper we report on a study of the effects of butanedione on the ($K^+ + H^+$)-ATPase from porcine gastric mucosa.

Materials and Methods

Isolation of ($K^+ + H^+$)-ATPase-containing membranes. A ($K^+ + H^+$)-ATPase-containing membrane fraction is isolated from porcine gastric mucosa accord-

ing to a procedure described by Forte et al. [6,16] with some modifications. Scrapings of fundic mucosa in 5 vols. buffer (113 mM mannitol, 37 mM sucrose, 0.2 mM EDTA, 5 mM Tris-HCl; pH 7.4) are homogenized with a Teflon-glass homogenizer (Braun) by 3 up-down strokes of the rotating pestle (300—500 rev./min). After filtration over four layers of surgical gauze, the homogenate is centrifuged at 20 000 × *g* for 20 min. 40 ml of the supernatant are layered over 8 ml of 37% (w/v) sucrose in 25 mM Tris-HCl (pH 7.4) in 48-ml screw-cap tubes and centrifuged at 100 000 × *g* for 30 min in an IEC B 60 ultracentrifuge (rotor 410).

The membrane fraction at the interface of the supernatant and sucrose layer is collected by means of suction and is diluted with 25 mM Tris-HCl (pH 7.4) to 15% (w/v) sucrose. The diluted membrane fraction is layered on a linear continuous sucrose gradient, ranging from 18 to 43% (w/v) sucrose in 25 mM Tris-HCl (pH 7.4) on a 55% (w/v) sucrose cushion, and is centrifuged overnight (16 h) in a zonal rotor (IEC B30 rotor, 47 000 rev./min). The gradient fractions are collected by pumping 55% (w/v) sucrose into the zonal rotor, monitoring the effluent at 254 nm, and collecting 20-ml fractions in a fraction collector. The membrane vesicles appear in the protein peak with a density of $d = 1.11$—1.13, and are collected by centrifuging for 1 h at 100 000 × g_{av} (IEC 410 rotor) after dilution with sucrose-free buffer. The pellet is resuspended in 0.25 M sucrose to a protein concentration of 1 mg/ml.

Butanedione inactivation studies. Reaction with butanedione is performed in a medium containing 125 mM sodium borate buffer (pH 7.0), 0.5 mg protein/ml and butanedione at the stated concentration. In the presence of a ligand (cation or nucleotide), the enzyme preparation is preincubated for 5 min at 37°C before addition of butanedione. After incubation at 37°C for a suitable time, 200-μl samples are removed. Separation of the enzyme preparation from the reaction mixture is performed by gel filtration at room temperature over a column (0.5 × 10 cm) of Sephadex G-25 coarse. The column is previously equilibrated in 125 mM borate buffer (pH 7.0), unless otherwise specified. 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 800 μl elution buffer, is used for enzyme assays. Control experiments in 125 mM borate buffer (pH 7.0) without butanedione are always included.

Enzyme assays. After addition of 20—50 μl samples to 400 μl medium at 0°C, the incubation mixture has the following final composition: 30 mM imidazole-HCl (pH 7.0), 5 mM disodium ATP, 5 mM $MgCl_2$, 0.1 mM ouabain and 20 mM KCl or choline chloride. For butanedione inactivation studies the imidazole buffer is replaced by 125 mM sodium borate (pH 7.0), which gives the same ATPase activity. After incubation for a suitable time at 37°C, the reaction is stopped by addition of 1 ml ice-cold 8.6% trichloroacetic acid. The released P_i is determined as described earlier [17]. Blanks in the choline chloride medium are run at 0°C. ($K^+ + H^+$)-ATPase activity is defined as the difference in activity between the K^+-containing and choline-containing media, run at 37°C. The difference in activity in the choline medium at 37°C and 0°C is called the Mg^{2+}-ATPase activity.

The related K^+-stimulated *p*-nitrophenyl phosphatase activity is assayed in the same way, except that ATP is replaced by 5 mM *p*-nitrophenyl phosphate and

the nitrophenol release is determined by reading the 410 nm absorbance after alkalinization [15].

Assay of marker enzymes. Cytochrome *c* oxidase, 5′-nucleotidase, ($Na^+ + K^+$)-ATPase and anion-sensitive ATPase activities are assayed as described by van Amelsvoort et al. [17].

Materials. Butanedione is obtained from Aldrich Europe (Beerse, Belgium): fresh dilutions are prepared before each experiment. All nucleotides are purchased from Boehringer (Mannheim, F.R.G.), ouabain Hepes, Tris, *p*-nitrophenyl phosphate, CDTA and imidazole from Merck (Darmstadt, F.R.G.).

Results

Isolation of ($K^+ + H^+$)-ATPase-containing membranes

The distribution of the various marker enzymes after centrifugation of the 20 000 × *g* supernatant on a 37% (w/v) sucrose layer obtained in a typical experiment is shown in Fig. 1. ($K^+ + H^+$)-ATPase (Fig. 1a) and K^+-pNPPase (Fig. 1b) are most pronounced in the interface fraction, whilst most of the Mg^{2+}-ATPase activity is lost (Fig. 1c). The low Mg^{2+}-ATPase activity in the interface fraction is accompanied by a very low cytochrome *c* oxidase activity (Fig. 1d), indicating a low mitochondrial contamination which accounts for most of the Mg^{2+}-ATPase activity.

A further purification of the ($K^+ + H^+$)-ATPase is achieved by subjecting the interface fraction to density gradient centrifugation. The distribution patterns of various marker enzymes are shown in Fig. 2. In the peak fraction, the ratio of ($K^+ + H^+$)-ATPase to Mg^{2+}-ATPase activity is now approx. 9. The specific ($K^+ + H^+$)-ATPase activity of the final enzyme preparation is 75—95 μmol P_i/mg protein per h. Gel electrophoresis shows a major band at 100 000 dalton, representing approx. 70% of the total protein content.

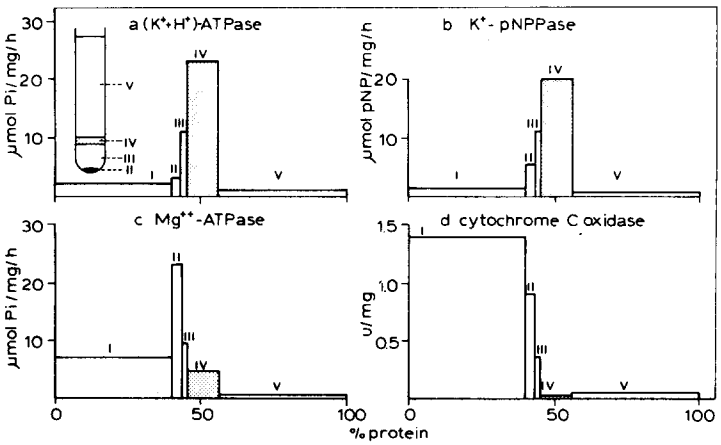


Fig. 1. Distribution of enzyme activities after fractionation of porcine gastric mucosa. (a) ($K^+ + H^+$)-ATPase, (b) K^+-pNPPase, (c) Mg^{2+}-ATPase and (d) cytochrome *c* oxidase activities are shown. Fractions II—V are explained by the inset: fraction I is the sediment from the preceding 20 min, 20 000 × *g* centrifugation.

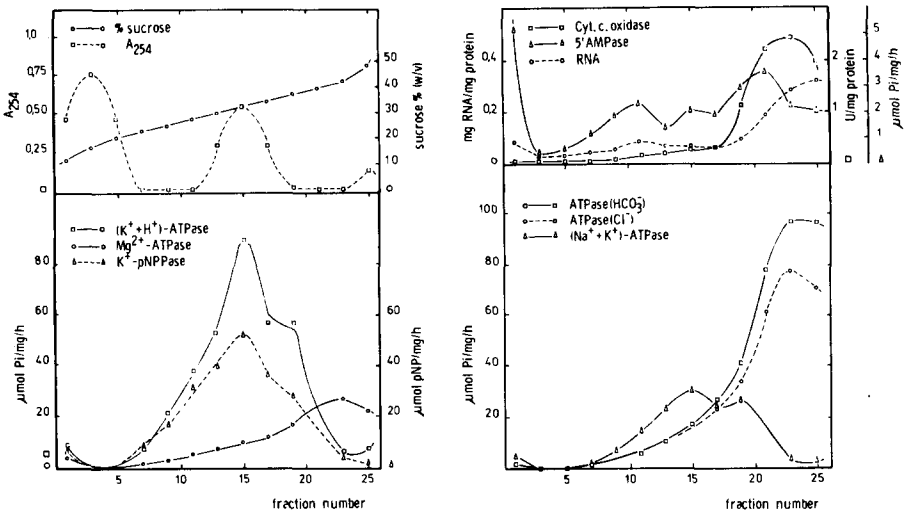


Fig. 2. Enzyme distribution pattern after density gradient centrifugation. The interface fraction (fraction IV in Fig. 1) is centrifuged (16 h, 47 000 rev./min, IEC B30 rotor) on a linear continuous sucrose gradient, ranging from 18 to 43% (w/v) sucrose on a 55% (w/v) sucrose cushion.

Properties of (K^+ + H^+)-ATPase

Fig. 3. shows the pH-dependence of the (K^+ + H^+)-ATPase and Mg^{2+}-ATPase activities. (K^+ + H^+)-ATPase activity has a pH optimum of 7.0, whilst Mg^{2+}-ATPase activity steadily increases over the measured pH range.

Fig. 4 presents Lineweaver-Burk plots for the activating effects of Tl^+, K^+,

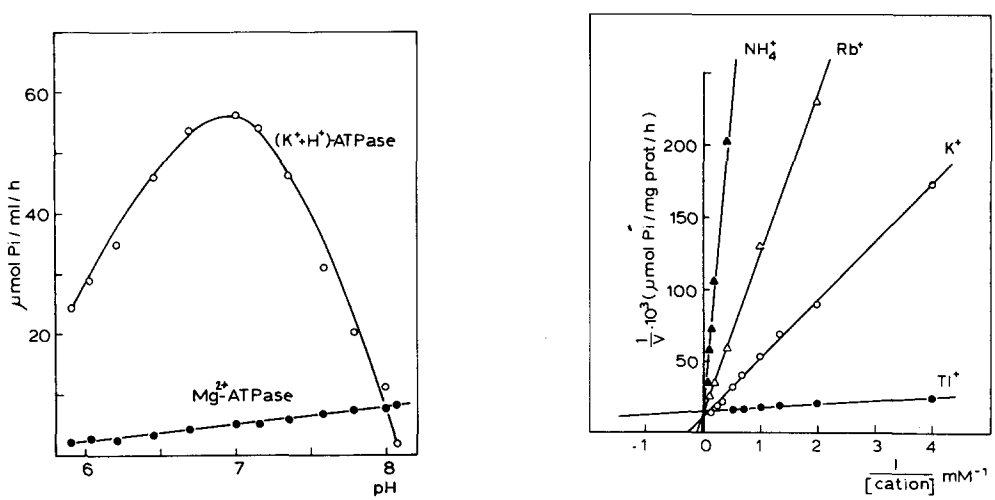


Fig. 3. (K^+ + H^+)- and Mg^{2+}-ATPase activities as a function of pH. Activities are determined as described in the text. The desired pH values were obtained by various mixtures of 96 mM imidazole and 96 mM HCl.

Fig. 4. Effects of monovalent cations on (K^+ + H^+)-ATPase activity. ATPase activity is determined as described in the text. Various concentrations of the indicated cations are added to the assay medium. Results are presented as Lineweaver-Burk plots. A boiled enzyme preparation is used as blank.

TABLE I

ASSOCIATION AND DISSOCIATION CONSTANTS FOR ACTIVATING MONOVALENT CATIONS

The gastric mucosal fraction is used for determination of K_A values from data as shown in Fig. 4. K_d and k_2/k_1 values for various monovalent cations are obtained by means of the Scruttion and Utter [22] equation from data as shown in Fig. 10.

Cation	Enzyme activation K_A	Effects on butanedione inactivation			
		ATP absent		1 mM ATP present	
		K_d	k_2/k_1	K_d	k_2/k_1
Tl^+	0.13 ± 0.02 (n = 2)	0.06	0.31	0.6	2.41
K^+	2.7 ± 0.2 (n = 4)	0.14	0.50	3.2	1.64
Rb^+	7.6 ± 0.2 (n = 2)	0.50	0.51	8.4	1.67
NH_4^+	26 ± 8 (n = 2)	0.94	0.74	—	—

Rb^+ and NH_4^+. Cs^+ shows only a slight activation at high concentration, whilst Na^+, Li^+ and choline$^+$ do not activate at all. The K_A values derived from results such as those in Fig. 4 are shown in Table I (2nd column). The activating sequence $Tl^+ > K^+ > Rb^+ > Cs^+$ has also been reported by others for both the ATPase and transport properties of gastric mucosal vesicles [4], as well as for the stimulation of the dephosphorylation of the phosphorylated enzyme by these cations [6]. The observed K_A value for K^+ (2.7 mM) is higher than the previously reported values of 0.3—1.0 mM [18,19]. This is probably due to the fact that the K_A value increases with decreasing pH [7], and our value is obtained at pH 7.0, whilst these other values were obtained at pH 7.5. The NH_4^+ concentration required for optimal activity is higher than that reported by Sachs [2], who states that NH_4^+ is equally active as K^+, whilst in our hands it is always less active than Rb^+.

Inactivation of ($K^+ + H^+$)-ATPase by butanedione

Preincubation of gradient purified ($K^+ + H^+$)-ATPase from porcine gastric mucosa with butanedione in borate buffer leads to inactivation of the enzyme. The degree of inactivation is dependent on a number of factors, such as butanedione concentrations, length of incubation, pH and presence of ligands in the medium. If incubation with butanedione is carried out in Tris-borate buffer instead of sodium borate buffer, inactivation is reduced (not shown), presumably due to a reaction of Tris with butanedione preventing arginine modification [20]. Treatment with butanedione also inactivates K^+-*p*-nitrophenylphosphatase activity, but in this case deviation from pseudo-first-order kinetics occurs beyond 40% inactivation, indicating more complex kinetics.

The pH of the preincubation buffer has a marked influence on the inactivation rate (Fig. 5). In the pH range 6.5—8.5 inactivation is most pronounced at pH values above pH 8.0, which has also been observed for butanedione inactivation of other enzymes [8,13,15]. Despite the larger inactivation at pH 8, we have chosen pH 7.0, the optimal pH for the enzyme activity (Fig. 3), for subsequent experiments.

Inactivation is reversible on removal of excess butanedione and borate. When

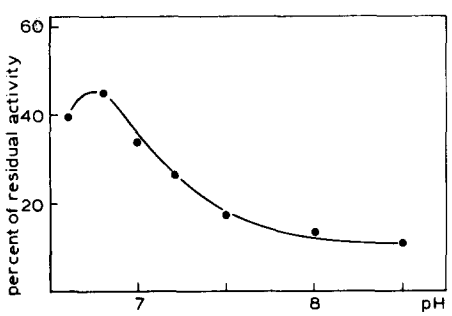
TABLE II

REVERSIBILITY OF THE INACTIVATION OF GASTRIC ($K^+ + H^+$)-ATPase BY BUTANEDIONE

The gastric mucosal vesicle fraction (0.5 mg protein/ml) is incubated for 20 min at 37°C in 125 mM sodium borate buffer (pH 7.0) containing 5 mM $MgCl_2$ and 0.5 mM butanedione. Aliquots are filtered through Sephadex G-25 columns, equilibrated and eluted with the indicated buffers. ($K^+ + H^+$)-ATPase activity is assayed, as described under Methods, immediately after gel filtration and 90 min later after standing at 37°C. Results are expressed as percent of ($K^+ + H^+$)-ATPase without butanedione treatment.

Buffer 125 mM, pH 7.0	Time after gel filtration (min)	
	0	90
Sodium borate	10	15
Imidazole-HCl	12	43
Tris-HCl	13	41
Hepes-NaOH	9	69

($K^+ + H^+$)-ATPase is modified to 10% of initial activity and gel filtration is performed with Hepes-NaOH buffer (pH 7.0) instead of borate buffer, the enzyme reactivates within 90 min at 37°C to 69% of control activity (Table II). Using imidazole-HCl or Tris-HCl instead of borate buffer also leads to reactivation of enzyme activity. The stabilization of the enzyme-butanedione complex



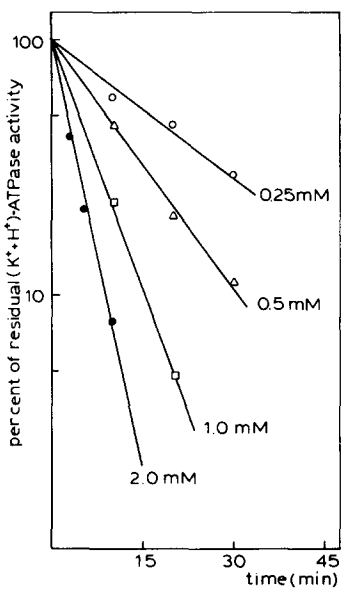


Fig. 5. Inactivation by butanedione as a function of pH. ($K^+ + H^+$)-ATPase preparation (0.5 mg/ml) is incubated for 20 min at 37°C with 0.5 mM butanedione in 125 mM borate buffer containing 5 mM $MgCl_2$, previously brought to the indicated pH values with 5 M NaOH. ($K^+ + H^+$)-ATPase activity is assayed as described under Methods and the activity is expressed as percent of control activity without butanedione.

Fig. 6. Inactivation by butanedione as a function of time. ($K^+ + H^+$)-ATPase preparation (0.5 mg protein/ml) is incubated at 37°C during the indicated times with various concentrations of butanedione in 125 mM sodium borate (pH 7.0), 5 mM $MgCl_2$. Enzyme activity is determined as described in the text, and is expressed as percent of control activity without butanedione.

by borate ions indicates that the inactivation is due to modification of essential arginine residues [8,10,11]. This reactivation with buffers other than borate does not occur when the eluted mixture is kept for 90 min at 0°C.

Fig. 6 shows that the reaction with butanedione exhibits pseudo-first-order kinetics up to 80% inactivation in the presence of 125 mM sodium borate buffer (pH 7.0) and 5 mM $MgCl_2$. The slope of a double-logarithmic plot of half-inactivation time against butanedione concentration gives the number of butanedione molecules reacting per enzyme molecule and involved in the inhibition of enzyme activity [21]. The slope of the line in Fig. 7 is 0.94, indicating that modification of one essential arginine residue is involved in the inactivation of the $(K^+ + H^+)$-ATPase by butanedione.

The apparent first-order reaction constant varies proportionally with the butanedione concentration (Fig. 8), indicating that the reaction obeys true second-order kinetics. The value of the second-order rate constant derived from Fig. 8 is 145 $min^{-1} \cdot M^{-1}$ in the presence of 5 mM Mg^{2+} and 52 $min^{-1} \cdot M^{-1}$ in the presence of 5 mM CDTA. These different rates suggest that the essential arginine residue is more exposed in the presence of 5 mM Mg^{2+}, and hence that Mg^{2+} induces a conformational change in the enzyme. This Mg^{2+} effect is concentration-dependent with an apparent K_A of 0.3 mM (not shown).

Protective effects of nucleotides on butanedione inactivation

The effects of ATP and other nucleotides on the butanedione inactivation of the enzyme have been studied. Fig. 9 shows the effects of ATP (right) and AMPPNP (left) with or without Mg^{2+}. ATP has a protective effect, which is stronger in the absence of Mg^{2+} than in its presence. The non-phosphorylating ATP-analogue, AMPPNP, also has a protective effect, but here the presence or

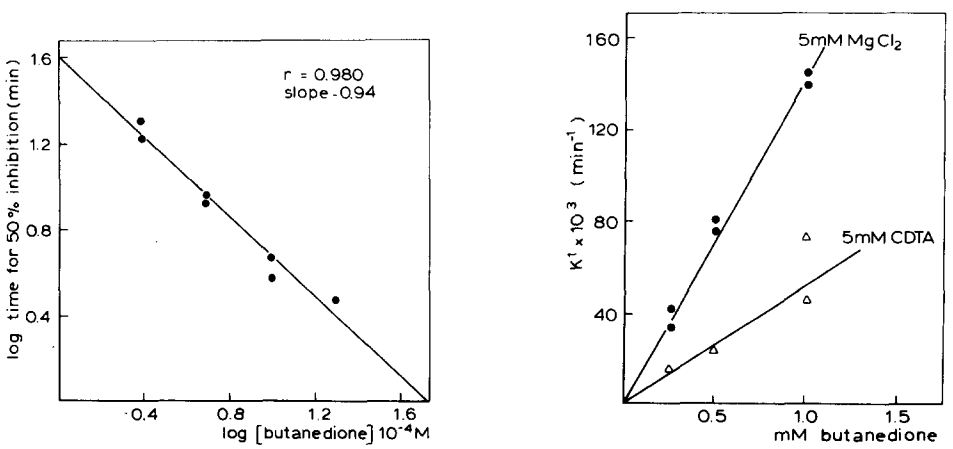


Fig. 7. Double-logarithmic plot of half-inactivation time vs. butanedione concentration. Treatment with butanedione is carried out in 125 mM sodium borate buffer (pH 7.0) containing 5 mM $MgCl_2$ (values from three separate experiments are plotted).

Fig. 8. Effect of butanedione concentration on pseudo-first-order rate constant. $(K^+ + H^+)$-ATPase preparation (0.5 mg/ml) is incubated at 37°C with various concentrations of butanedione in 125 mM sodium borate buffer, containing 5 mM $MgCl_2$ or 5 mM CDTA. Enzyme activity is assayed as described under Methods. Data from two experiments are plotted.

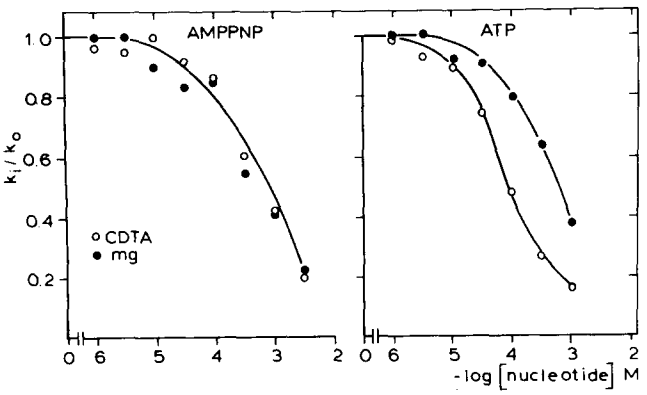


Fig. 9. Protective effects of ATP and AMPPNP against butanedione inactivation. ($K^+ + H^+$)-ATPase (0.5 mg/ml) is incubated at 37°C in 125 mM sodium borate buffer (pH 7.0) containing either 5 mM $MgCl_2$ or 5 mM CDTA and ATP and AMPPNP in the stated concentrations. Inactivation is stopped by gel filtration and the eluted enzyme is assayed. Results are expressed as the ratio of the pseudo first-order-inactivation rate constants in the absence (k_0) or the presence (k_i) of nucleotide.

absence of Mg^{2+} makes little or no difference. ADP and deoxy-ATP also have a protective effect, which is decreased by addition of Mg^{2+} (Table III). Other nucleotides (CTP, GTP, ITP) show hardly any protection, even at rather high concentrations, which parallels the low activity of the enzyme for these nucleotides as substrate (Table III, 2nd column).

Effects of activating cations on butanedione inactivation

The effects of the activating monovalent cations on butanedione inactivation in the absence of ATP are shown in Fig. 10. It is obvious that Tl^+, K^+, Rb^+ and NH_4^+ can all protect against butanedione inactivation, but in decreasing order of

TABLE III

PROTECTIVE EFFECT OF NUCLEOTIDES ON INACTIVATION BY BUTANEDIONE

($K^+ + H^+$)-ATPase is incubated for 20 min at 37°C in 125 mM sodium borate buffer (pH 7.0) containing 0.5 mM butanedione, 1 mM of the indicated compounds and either 5 mM $MgCl_2$ or 5 mM CDTA, after preincubation for 5 min before butanedione was added. Results are expressed as percent of inhibition obtained without nucleotide (n = 2). The K^+-stimulated hydrolysis for each nucleotide (5 mM) as a percentage of the hydrolysis of ATP is shown in column 2.

Nucleotide added (1 mM)	Activity as substrate %	Percent inhibition (5 mM)	
		$MgCl_2$	CDTA
None	—	100	100
ATP	100	59 ± 2.5	24 ± 5.0
ADP	1	61 ± 5.0	42 ± 3.8
deoxy-ATP	62	75 ± 2.5	43 ± 2.5
AMPPNP	0	67 ± 3.8	57 ± 1.3
AMPPCP	0	92 ± 2.5	87 ± 2.5
AMP	1	92 ± 2.5	100 ± 5.0
CTP	17	85 ± 3.8	91 ± 3.8
GTP	0	83 ± 3.8	89 ± 2.5
ITP	0	86 ± 2.5	89 ± 1.3

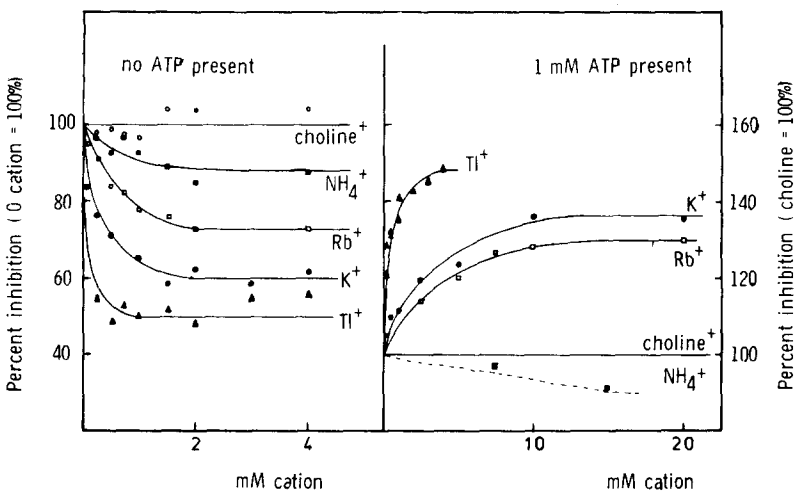


Fig. 10. Protective effects of activating monovalent cations on inactivation by butanedione. $(K^+ + H^+)$-ATPase preparation (0.5 mg protein/ml) is incubated at 37°C in 125 mM sodium borate (pH 7.0) containing 1 mM CDTA, butanedione and activating monovalent cations in the stated concentrations in the absence (left) or presence (right) of 1 mM ATP. Enzyme activity is determined as described in the text. Results are expressed as percent of the inhibition in the absence of cation (inhibition = 100%). In the absence of ATP, 0.5 mM butanedione and 15 min incubation; in the presence of ATP, 2 mM butanedione and 40 min incubation were used in order to obtain inactivation percentages comparable to those without activating cation.

effectiveness. Non-activating cations like Cs^+, Li^+, Na^+ and choline show hardly any effect. Both the rate of inactivation and the final level of protection depend on the cation used.

The dissociation constant, K_d, for the enzyme-cation complex has been calculated by means of the following equation derived by Scrutton and Utter [22]:

$$\frac{k_i}{k_0} = \frac{k_2}{k_1} + K_d \cdot \frac{1 - \frac{k_i}{k_0}}{[L]}$$

where k_i and k_0 represent the pseudo first order inactivation rate constants in the presence and absence of the monovalent cation, K_d is the dissociation constant for the enzyme-cation complex, k_1 the rate constant for the reaction between free enzyme and butanedione, k_2 that between the enzyme-cation complex and butanedione and [L] the concentration of the cation. By plotting k_i/k_0 vs. $(1 - k_i/k_0)/[L]$, the value of K_d can be deduced from the slope for each cation (Table I, 3rd column). The protecting effect of the monovalent cations is clearly shown by the low k_2/k_1 values (Table I, 4th column).

When these experiments are repeated in the presence of ATP, there is a striking reversal of the cation effects on butanedione inactivation, but again in the same order of effectiveness: $Tl^+ > K^+ > Rb^+ > NH_4^+$ (Fig. 10). Only NH_4^+ behaves somewhat abnormally, inasmuch as it has less effect than choline. The values of K_d derived from these curves are shown in Table I, 5th column. They are close to the K_A values for the activating effect of the cations on the ATPase

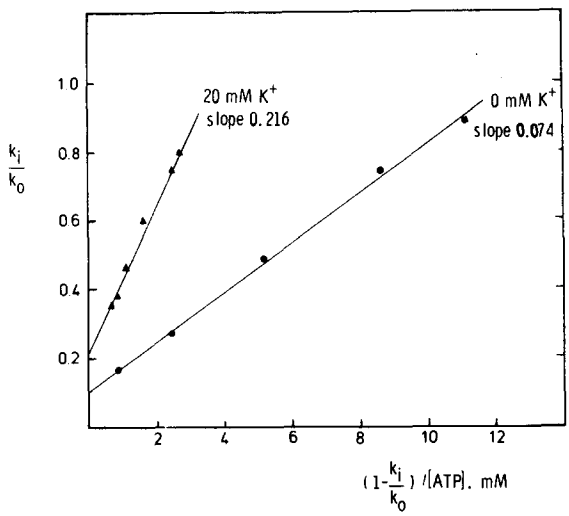


Fig. 11. Effect of K^+ on the dissociation constant of the enzyme-ATP complex. Data, averaged from two to four experiments similar to those shown in Fig. 9 (right), are plotted according to the Scrutton and Utter equation [22].

activity. The rather high k_2/k_1 values (Table I, 6th column) indicate that, in this case, the inactivation by butanedione increases upon addition of activating monovalent cations.

These experiments suggest that the activating monovalent cations induce a conformational change, which leads to decreased exposure of the essential arginine residue. This leads in turn to a decreased binding of ATP and hence to the increased inactivation in its presence. In order to test this last conclusion, we have determined the dissociation constant, K_d, for ATP by observing butanedione inactivation in the presence of 0 and 20 mM KCl at different concentrations of ATP (Fig. 11). The value of K_d, calculated by the method of Scrutton and Utter [22] inserting for [L] the ATP concentration, is 216 μM in the presence and 74 μM in the absence of K^+, which confirms our conclusion.

Discussion

The membrane vesicles prepared from porcine gastric mucosa are quite similar to those prepared by other investigators [1—5]. This is true for the high ($K^+ + H^+$)-ATPase activity as well as for the capacity for ATP-dependent proton uptake (not shown). The isolated method used by us gives quite satisfactory results. Use of ficoll-sucrose gradients as described by Saccomani et al. [23] enhances the degree of stimulation by the ionophores, valinomycin and gramicidin, but also increases the Mg^{2+}-ATPase activity in the peak fractions (not shown). The pH optimum of 7.0 for the ($K^+ + H^+$)-ATPase activity is in agreement with recent findings of Lee et al. [7]. The K_A value for K^+ is in fair agreement with values previously reported by others, when the difference in pH at which the values were determined is kept in mind [7]. The final specific activity of the ($K^+ + H^+$)-ATPase activity in the isolated gradient fractions (75—

95 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$) is only slightly lower than the activity of 110 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ obtained by Saccomani et al. [23] after free-flow electrophoresis.

The inhibition of the $(K^+ + H^+)$-ATPase activity by butanedione is reversible, but can be stabilized by the borate anion. This indicates modification of arginine groups [8,10,11]. The pH-dependence of the reaction supports this conclusion. The kinetic behaviour of the butanedione reaction suggests that modification of one essential arginine residue is responsible for the inactivation of enzyme activity. This does not, of course, exclude that many more are modified, but these would then be non-essential for the activity of the enzyme.

The study of the effects of various ligands on the butanedione reaction has yielded useful further information on the role of the essential arginine group and on conformational states of the enzyme. The faster inactivation in the presence of Mg^{2+} indicates a conformational change induced by Mg^{2+}, leading to increased exposure of the arginine group. The ability of ATP to protect against butanedione inactivation suggests that the essential arginine group is located in the ATP binding center. Such a role for the positively-charged arginine residue has been proposed for many other enzyme systems, which are dependent on negatively-charged substrates or co-factors.

In the presence of Mg^{2+} and ATP there occurs phosphorylation of the enzyme [6]. This could explain why ATP protects less against inactivation by butanedione in the presence of Mg^{2+} than in its absence. One would expect that AMPPNP, an analogue which cannot be hydrolyzed, would give better protection in the presence of Mg^{2+} than in its absence, since the essential arginine residue is more exposed in the former case. There is, however, no clear effect observable. It could be that the increased exposure of the arginine group would favour the binding of AMPPNP and of butanedione about equally. It might also be that AMPPNP antagonizes the effect of Mg^{2+}. The fact that the protecting effect of ADP is less in the presence of Mg^{2+} than in its absence would fit a scheme in which Mg^{2+} induces a release of ADP from the enzyme, thus vacating the ATP binding site for another ATP molecule. Binding studies with ATP, ADP and AMPPNP in the presence and absence of Mg^{2+} are needed to settle these matters.

The only other nucleotide, which shows a significant Mg^{2+} effect on its protection is deoxy-ATP. This suggests that the structure of the sugar moiety of the nucleotide is not very critical for binding or phosphorylation. The inability of CTP, GTP and ITP to protect against butanedione inactivation agrees with their low activity as substrates for the enzyme.

The activating monovalent cations seem to induce a conformational change in the enzyme, which is opposed to that induced by Mg^{2+}. In the absence of ATP these cations protect against butanedione inactivation, indicating reduced exposure of the arginine group. In the presence of ATP they show a reverse effect: they increase the inactivation found with ATP alone. This is understandable from the decreased exposure of the arginine residue in this conformation: binding of ATP is decreased and hence its protecting effect is diminished. In agreement with this, we find that the dissociation constant, K_d, of the enzyme-ATP complex is about three times as high in the presence of 20 mM K^+ as in its absence. Studies on $(Na^+ + K^+)$-ATPase with the sulfhydryl reagent, 7-chloro-4-

nitrobenzo-2-oxa-1,3-diazole, indicate that K^+ also decreases the binding of ATP in this enzyme [24]. Conversely, studies with *N*-ethylmaleimide on this enzyme suggest that ATP decreases the apparent affinity for K^+ [25]. Occurrence of a similar interaction between K^+ and ATP in the case of (K^+ + H^+)-ATPase could explain our observation that the dissociation constants for the effect of the activating monovalent cations in the absence of ATP are much lower than those obtained in its presence (Table I, columns 3 and 5).

The same order of effectiveness ($Tl^+ > K^+ > Rb^+ > NH_4^+$) for activation of the enzyme and for lowering the affinity for ATP (with more or less quantitative agreement between the K_A and K_d values: Table I, columns 2 and 5) agree with their order of effectiveness in dephosphorylating the phosphorylated enzyme [6]. Hence, it appears that these three phenomena are interrelated and that the monovalent cation-induced conformation of the enzyme favours its dephosphorylation. Our observation that the final level of the cationic effect is dependent on the cation used, indicates that greater activation goes together with stronger expression of the conformational change. It is paralleled by the findings of Ray and Forte [6], showing that the level of the remaining phosphorylated intermediate depends on the monovalent cation present.

The observed effects of various ligands on the butanedione modification of the essential arginine group involved in substrate binding appears to suggest that the enzyme can occur in at least two conformational states:

1. An Mg^{2+}-induced state, which favours exposure of an essential arginine residue and which is involved in ATP binding, phosphorylation and possibly release of ADP.

2. A monovalent cation-induced state which favours dephosphorylation. This would imply that there is a great similarity in the mechanism of action between the (K^+ + H^+)-ATPase and the (Na^+ + K^+)-ATPase systems.

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