Biochimica et Biophysica Acta, 482 (1977) 213—227

BBA 68136

AN ESSENTIAL ARGININE RESIDUE IN THE ATP-BINDING CENTRE OF ($Na^+ + K^+$)-ATPase *

J.J.H.H.M. DE PONT, B.M. SCHOOT, A. VAN PROOIJEN-VAN EEDEN and S.L. BONTING

Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)

(Received September 6th, 1976)

Summary

1. Incubation of purified ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase EC 3.6.1.3) from rabbit kidney outer medulla with butanedione in borate buffer leads to reversible inactivation of the ($Na^+ + K^+$)-ATPase activity.

2. The reaction shows second-order kinetics, suggesting that modification of a single amino acid residue is involved in the inactivation of the enzyme.

3. The pH dependence of the reaction and the effect of borate ions strongly suggest that modification of an arginine residue is involved.

4. Replacement of Na^+ by K^+ in the butanedione medium decreases inactivation.

5. ATP, ADP and adenylyl imido diphosphate, particularly in the presence of *trans*-1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetic acid to complex Mg^{2+}, protect the enzyme very efficiently against inactivation by butanedione.

6. The ($Na^+ + Mg^{2+}$)-dependent phosphorylation capacity of the enzyme is inhibited in the same degree as the ($Na^+ + K^+$)-ATPase activity by butanedione.

7. The K^+-stimulated *p*-nitrophenylphosphatase activity is much less inhibited than the ($Na^+ + K^+$)ATPase activity.

8. The ATP stimulation of the K^+-stimulated *p*-nitrophenylphosphatase activity is inhibited by butanedione to the same extent as the ($Na^+ + K^+$)-ATPase activity.

9. Modification of sulfhydryl groups with 5,5'-dithiobis(2-nitrobenzoic acid) protects partially against the inactivating effect of butanedione.

10. The results suggest that an arginine residue is present in the nucleotide binding centre of the enzyme.

---

* This paper forms part XL of "Studies on Na-K-activated ATPase".

Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetic acid; Nbs_2, 5,5'-dithiobis(2-nitrobenzoic acid); HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate.

Introduction

Our understanding of the molecular mechanism of $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is still very incomplete. This is partly due to the difficulty in the past in obtaining highly purified preparations of the enzyme and partly to the complex kinetics of $(Na^+ + K^+)$-ATPase (for reviews see refs. 1—4). One approach to obtaining information on the mechanism of this enzyme is through chemical modification of particular residues in the enzyme complex by means of group-specific reagents. With the aid of *N*-ethylmaleimide and similar reagents evidence has been obtained for the presence of sulfhydryl groups in the active center of the enzyme [5—9].

In the last few years reagents such as butanedione and phenylglyoxal, which react specifically with arginine residues, have often been applied in studies of enzymes. Riordan et al. and others have shown that in enzymes, in which anionic substrates [10—15] and cofactors [16—20] play a role, arginine residues are often involved in the binding of the negatively charged substrate or cofactor. Very recently such a role has been established in a bicarbonate-sensitive ATPase in *Escherichia Coli* [21] and in mitochondrial ATPase from beef heart and rat liver [22].

In this paper we report a study of the effect of the arginine reagent butanedione on highly purified $(Na^+ + K^+)$-ATPase from rabbit kidney outer medulla. We find that reaction of $(Na^+ + K^+)$-ATPase with butanedione leads to reversible inactivation of $(Na^+ + K^+)$-ATPase. The K^+-stimulated *p*-nitrophenylphosphatase activity, which is always associated with the $(Na^+ + K^+)$-ATPase activity, is much less inactivated by this reagent. This suggests that arginyl residues play a particular role in the $(Na^+ + K^+)$-ATPase system.

Materials and Methods

Enzyme preparation. $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) is purified from rabbit kidney outer medulla according to Jørgensen [23]. In order to remove any ATP present in the enzyme preparation [23] 10 ml of the ATPase incubation medium A (ref. 1, p. 261) without ATP is added to 2 ml of the enzyme preparation. After incubation at 37°C for 15 min the enzyme is sedimented by centrifugation for 10 min at 300 000 × *g* in an A321 rotor of an IEC B 60 ultracentrifuge. In order to remove adjacent ions the enzyme is twice washed with imidazole (25 mM), pH 7.5. The enzyme is stored at −20°C in the latter buffer to which 0.25 M sucrose is added. The activity of the preparations is 1300—2000 μmol ATP/h per mg protein. The K^+-stimulated *p*-nitrophenylphosphatase activity is 300—400 μmol *p*-nitrophenol formed/h per mg protein. The preparations are free from ouabain-insensitive Mg^{2+}-ATPase activity and with SDS gel electrophoresis the two normally appearing bands [2,3] comprise at least 95% of all protein.

Reaction with butanedione. $(Na^+ + K^+)$-ATPase (normally 30 μg/ml) is incubated at 25°C in sodium borate buffer (50 or 125 mM, pH 7.5) to which normally 5 mM $MgCl_2$ is added. To this medium various concentrations of butanedione, present in the same buffer, are added. At the indicated times samples are removed for the assay of $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-

nitrophenylphosphatase activities. Since the degree of inhibition differs for various enzyme preparations, a range of concentrations of butanedione must be used for each new batch of enzyme in order to determine the sensitivity of the preparation.

Enzyme assays. ($Na^+ + K^+$)-ATPase activity is determined, after preincubation with butanedione, essentially as described by Bonting (ref. 1, p. 261). Aliquots (20 µl) of the preincubation mixture (or appropriate dilutions in deionized distilled water) are mixed with 300 µl of medium A (55 mM Na^+, 5 mM K^+, 2 mM ATP, 2 mM Mg^{2+}, 0.1 mM EDTA, 100 mM Tris · HCl, pH 7.5) and medium E (medium A without K^+, but with 10^{-4} M ouabain), respectively. After 10—20 min incubation at 37°C free inorganic phosphate is determined. The difference in the activities in the two media is taken as the ($Na^+ + K^+$)-ATPase activity.

The K^+-stimulated *p*-nitrophenylphosphatase activity of the preparation is assayed, after preincubation with butanedione, by mixing 20-µl aliquots of the preincubation mixture (or appropriate dilutions in deionized distilled water) with 300 µl of media P or Q, respectively. Medium P contains 10 mM imidazole (pH 7.4), 5 mM $MgCl_2$, 10 mM KCl, 1 mM CDTA, 5 mM *p*-nitrophenylphosphate. Medium Q contains no KCl but 10^{-4} M ouabain. After incubation at 37°C for 20 min 2 ml 0.5 M NaOH is added and the extinction at 410 nm is measured. The difference in activities between media P and Q is taken as the K^+-stimulated *p*-nitrophenylphosphatase activity.

The Na^+-dependent phosphorylation of the ($Na^+ + K^+$)-ATPase is carried out after the preincubation with butanedione. To 25 µl of the preincubation mixture (which is five times diluted with 125 mM borate buffer, pH 7.5) 75 µl of a solution containing 2 µCi [γ-^{32}P]ATP and other ions is added, resulting in the following final concentrations: 17 µM [γ-^{32}P]ATP, 5 mM Mg^{2+}, 138 mM Na^+, 125 mM borate buffer (pH 7.5). After 3 s incubation at 37°C the reaction is stopped by adding 2 ml 5% trichloroacetic acid. A sample, to which trichloroacetic acid is added before addition of ATP, serves as a blank. The mixtures are quantitatively transferred to a Selectron filter (1.2 µM; Schleicher and Schüll, Dassel, G.F.R.) by means of two additional volumes of 5% trichloroacetic acid. The filters are washed four times with 5 ml 5% trichloroacetic acid and are dissolved in 10 ml Aquasol (N.E.N. Boston, Mass., U.S.A.). The radioactivity is counted in a liquid scintillation counter (Philips LSA).

Reactivation studies. Enzyme, which has been approx. 80% inactivated by butanedione, is chromatographed at 4°C on a column (0.5 × 10 cm) of Sephadex G-25. The column is equilibrated in either 50 mM borate, veronal, HEPES or Tris buffer, all at pH 7.5, and the same buffers are used for elution. Control samples, not treated with butanedione, are treated in the same way. The activities of the filtrates are measured before, immediately after and 90 min after passage through the column.

Protection of sulfhydryl groups. Modification of sulfhydryl groups is carried out by adding 10 µl 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) in 30 mM acetate buffer (pH 5.3) to 90 µl of a ($Na^+ + K^+$)-ATPase suspension (1 mg/ml) in 125 mM sodium borate buffer (pH 7.5). After treatment with butanedione the ($Na^+ + K^+$)-ATPase suspension is first chromatographed in borate buffer as described above. After 1 h at 25°C the reaction is complete, and the enzyme is

sedimented by centrifugation for 15 min at 10 000 × *g*. The absorbance is measured spectrophotometrically at 412 nm, using a blank in which Nbs_2 has been replaced by the buffer [24]. The number of modified sulfhydryl groups is calculated with cysteine as a standard.

In some experiments 2 mM butanedione is added to some of the samples after treatment with Nbs_2 and the incubation at 25°C is extended by 30 min. Then dithioerythritol (to a final concentration of 10 mM) is added to half of the samples and an equal volume of water to the others. After incubation for another hour at 25°C the $(Na^+ + K^+)$-ATPase activity is determined. In some experiments a gel filtration step, as described in the previous section, is applied after the dithioerythritol treatment and the enzyme assay is carried out in the usual way.

Amino acid analysis. Samples of the reaction medium containing $(Na^+ + K^+)$-ATPase, preincubated for various times with butanedione, are added to excess 6 M HCl. After centrifugation for 30 min at 5000 × *g* the pellet is taken up in 6 M HCl and digested for 22 h at 110°C in vacuo. Amino acid analysis is carried out on a Rank Hilger Chromaspek amino acid analyzer.

Free amino groups are determined fluorimetrically using the Fluram reagent according to Böhlen et al. [25] with the exception that 1% SDS is added to all samples before adding Fluram. Protein is determined by the method of Lowry et al. [26] after precipitation with trichloroacetic acid as described by Jørgensen [27]. Bovine serum albumin is used as a standard.

Materials. 2,3-Butanedione is obtained from Aldrich Europe (Beerse, Belgium); fresh dilutions are prepared before each experiment. Nbs_2 is purchased from B.D.H. Chemicals Ltd. (Poole, England). Dithioerythritol is from Sigma (St. Louis, Mo., U.S.A.). [γ-^{32}P]ATP (spec. act. about 1200 Ci/mol) is supplied by The Radiochemical Centre (Amersham, England). Fluram (fluorescamine) is obtained from Hoffman-La Roche (Nutley, N.J., U.S.A.). All nucleotides are from Boehringer (Mannheim, G.F.R.). HEPES, ouabain, *p*-nitrophenylphosphate, EDTA and CDTA are from Merck (Darmstadt, G.F.R.).

Results

Effect on $(Na^+ + K^+)$-ATPase activity

Preincubation of purified $(Na^+ + K^+)$-ATPase, prepared from rabbit kidney outer medulla, with butanedione in borate buffer leads to inactivation of the enzyme. The degree of inactivation is dependent on a number of factors, such as concentration of butanedione, time of incubation, pH and concentration of the borate buffer and the presence of other ions in the medium.

Fig. 1 shows that in the presence of 50 mM borate buffer (pH 7.5) and 5 mM Mg^{2+} the reaction exhibits pseudo first-order kinetics up to 90% inactivation at each of the concentrations of butanedione used. The apparent first-order rate constant varies proportionally with the concentration, indicating that the reaction obeys second-order kinetics. The second-order rate constant, which is equal to the apparent first-order rate constant divided by the butanedione concentration, amounts to 19 $min^{-1} \cdot M^{-1}$ under the conditions of the experiment. The fact that the reaction shows second-order kinetics indicates that inactivation of the enzyme is due to the reaction of one residue in the $(Na^+ +$

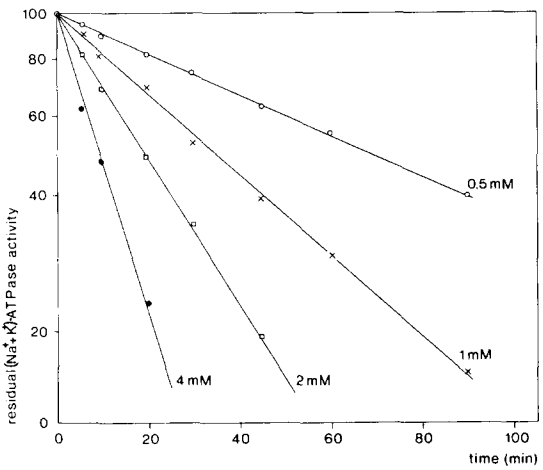

Fig. 1. Inactivation by butanedione as a function of time. $(Na^+ + K^+)$-ATPase (30 μg/ml) is preincubated at 25°C during the indicated times with various concentrations of butanedione in 125 mM sodium borate buffer, containing 5 mM $MgCl_2$ (pH 7.5). $(Na^+ + K^+)$-ATPase is determined as described under Materials and Methods. Enzyme activity is expressed as percent of control activity without butanedione.

K^+)-ATPase complex with one molecule of butanedione. This does not exclude the possibility that more than one vital arginine residue is present in the enzyme, each of which can cause a complete inactivation of the $(Na^+ + K^+)$-ATPase activity.

Replacement of Na^+ in the preincubation medium by K^+ leads to a significant reduction in the degree of inactivation (Table I). Fig. 2 shows that the change in sensitivity towards butanedione already occurs when only a small part of Na^+ is replaced by K^+, in 50% change sensitivity being found at 0.5 mM K^+ (and 14.5 mM Na^+). This suggests that the change is due to an effect of K^+ on the enzyme and thus that a K^+-sensitive site is involved. Replacement of Mg^{2+} by CDTA has no significant effect on the degree of inhibition by butanedione. However, when the $(Na^+ + K^+)$-ATPase preparation has not been washed to remove ATP from the enzyme preparation, a significantly higher degree of inactivation is found in the presence of Mg^{2+} than in that of CDTA. This can be explained by the fact that ATP protects the enzyme against inactivation (vide infra) and that Mg^{2+} remove bound ATP from its binding site.

TABLE I

EFFECT OF CATIONS ON INACTIVATION OF $(Na^+ + K^+)$-ATPase BY BUTANEDIONE

$(Na^+ + K^+)$-ATPase (30 μg/ml) is incubated for 30 min at 25°C in 50 mM sodium or potassium borate buffer (pH 7.5), containing 4 mM butanedione and 5 mM $MgCl_2$. The monovalent cation concentration in the buffer is 15 mM. $(Na^+ + K^+)$-ATPase activity is determined as described in Materials and Methods. Results are given as means with S.E. of three experiments.

Cations present	Residual $(Na^+ + K^+)$-ATPase activity (percent of control)
$Mg^{2+} + Na^+$	14 ± 1.8
$Mg^{2+} + K^+$	37 ± 1.9

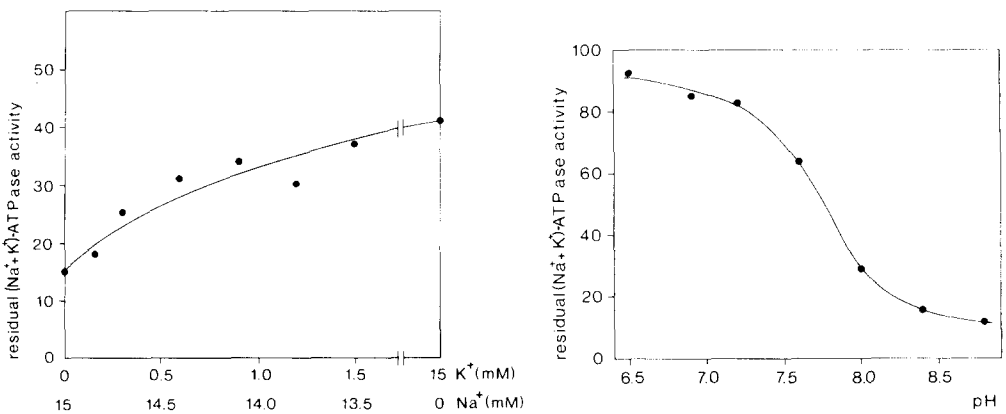

Fig. 2. Effect of replacement of Na^+ by K^+ on the inactivation by butanedione. ($Na^+ + K^+$)-ATPase (30 μg/ml) is preincubated during 30 min at 25°C with 4 mM butanedione in 50 mM borate buffer (pH 7.5), containing 5 mM Mg^{2+} and concentrations of Na^+ and K^+ as indicated in the figure. ($Na^+ + K^+$)-ATPase is determined as described under Materials and Methods. Enzyme activity is expressed as percent of control activity without butanedione.

Fig. 3. Inactivation by butanedione as a function of pH. ($Na^+ + K^+$)-ATPase (30 μg/ml) is preincubated during 30 min at 25°C with 2 mM butanedione in 125 mM sodium borate buffer containing 5 mM $MgCl_2$, previously brought to the indicated pH. ($Na^+ + K^+$)-ATPase is determined as described under Materials and Methods. Enzyme activity is expressed as percent of control activity without butanedione.

The pH of the preincubation buffer also has a marked influence on the degree of inactivation. In the pH range of 6.5—8.8 inactivation is most pronounced at pH values above 7.5 (Fig. 3). This pH dependence is qualitatively similar to that observed for other enzymes [10,16,18]. This effect has been interpreted as an effect on the borate buffer or on another functional group in the active center rather than as a change in the activity of the pK value of an arginine residue [10,16]. Despite the larger inactivation at pH > 8, we have chosen a more physiological pH of 7.5 for subsequent experiments. The degree of inactivation by butanedione also varies with the borate concentration (Fig. 4). Maximal inactivation is observed in the presence of 25 mM borate, whereas the effect decreases somewhat at higher borate concentrations. At 200 mM borate the effect of butanedione is markedly reduced. This behaviour agrees qualitatively with that observed for other enzymes [10,13,18,19].

Reversibility of the inactivation is tested by gel filtration of the inactivated enzyme complex on Sephadex G-25, equilibrated with various buffers. The sample eluted with 50 mM borate buffer is not reactivated, whereas the activities of the other samples, which have been eluted with 50 mM veronal, Tris or HEPES buffers, are partly restored (Table II). This indicates that the butanedione · enzyme complex is stabilized by borate ions.

The effect of ATP and other nucleotides on inactivation by butanedione has been determined. In the presence of Mg^{2+} the inactivation by butanedione is partially prevented by ATP (Fig. 5A, upper curve), but the concentration dependence is slight and there is a large variability in the degree of inactivation. When Mg^{2+} is replaced by an equal concentration of CDTA, the protective effect of ATP is more pronounced and is noticeable at very low concentrations

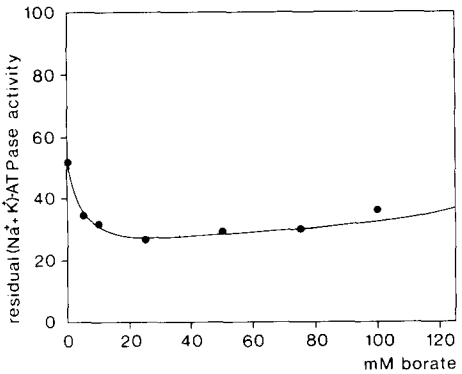

Fig. 4. Inactivation by butanedione as a function of borate concentration. $(Na^+ + K^+)$-ATPase (30 μg/ml) is preincubated during 30 min at 25°C with 2 mM butanedione in 125 mM buffer, containing 5 mM $MgCl_2$. Mixtures of borate and HEPES buffer (pH 7.5) are used, their total concentration being maintained at 125 mM. $(Na^+ + K^+)$-ATPase is determined as described under Materials and Methods. Enzyme activity is expressed as percent of control activity without butanedione.

(Fig. 5A, lower curve), while the variability in the results is much smaller. ADP also protects against inactivation by butanedione (Fig. 5B). Of various phosphate compounds applied in low concentrations (10 μM) in the presence of CDTA, only ATP, ADP and to a lesser degree the ATP analogue adenylyl imido diphosphate affect the inactivation by butanedione (Table III). For all three nucleotides the protective effect is much more pronounced in the absence of Mg^{2+}.

Preincubation with butanedione does not only lead to a reduction in $(Na^+ + K^+)$-ATPase activity, but also to a reduction in the $(Na^+ + Mg^{2+})$-dependent phosphorylation. There is no significant difference in the effects on these two activities (Table IV).

Effect on K^+-stimulated p-nitrophenylphosphatase

The K^+-stimulated *p*-nitrophenylphosphatase activity, which is thought to

TABLE II

REVERSIBILITY OF INACTIVATION OF $(Na^+ + K^+)$-ATPase BY BUTANEDIONE

$(Na^+ + K^+)$-ATPase (1.56 mg/ml) is incubated for 30 min at 25°C in 50 mM sodium borate buffer (pH 7.5) containing 5 mM $MgCl_2$ and 5 mM butanedione. Aliquots are filtered through Sephadex G-25 columns with the indicated buffer (50 mM, pH 7.5). $(Na^+ + K^+)$-ATPase activity is assayed as described under Materials and Methods before, immediately after and 1.5 h after gel filtration. Results are expressed as percent of controls without butanedione. Activity before gel filtration is 18% of the control.

Buffer used	$(Na^+ + K^+)$-ATPase activity (percent of control) time after fel filtration	
	0 h	1.5 h
Borate	15	17
Veronal	22	63
HEPES	28	88
Tris · HCl	23	65

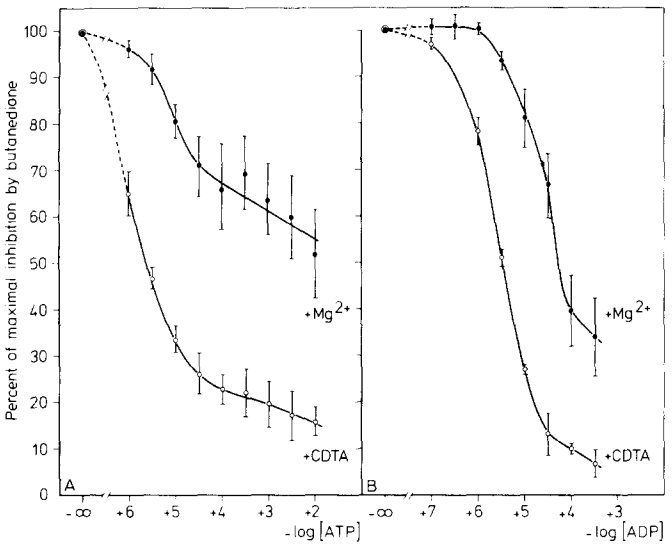

Fig. 5. Protective effect of ATP (A) and ADP (B) against inactivation by butanedione. $(Na^+ + K^+)$-ATPase (30 μg/ml) is preincubated during 30 min at 25°C in 50 mM sodium borate buffer (pH 7.5) containing either 5 mM Mg^{2+} or 5 mM CDTA, 4 mM butanedione and ATP or ADP in the indicated concentrations expressed in mol/l. $(Na^+ + K^+)$-ATPase is determined as described under Materials and Methods. Results are expressed as percent of inhibition obtained in the absence of added nucleotide. Results represent means and S.E. of three experiments.

represent the phosphatase step in the $(Na^+ + K^+)$-ATPase reaction mechanism, is much more resistant to inactivation by butanedione than the $(Na^+ + K^+)$-ATPase activity. Fig. 6 shows the effect of various concentrations of butanedione on the two enzyme activities, measured after preincubation periods of 30 and 120 min, at 25°C. This difference in sensitivity towards butanedione is

TABLE III

EFFECT OF PHOSPHATE COMPOUNDS ON INACTIVATION OF $(Na^+ + K^+)$-ATPase BY BUTANEDIONE

$(Na^+ + K^+)$-ATPase (30 μl/mg) is preincubated for 30 min at 25°C in 50 mM sodium borate buffer (pH 7.5) containing 5 mM CDTA, 8 mM butanedione and 10 μM of the indicated compounds. $(Na^+ + K^+)$-ATPase determination are carried out as described under Materials and Methods. Results are expressed as percent of the inhibition obtained in the absence of added substance. Results represent means and S.E. of three experiments.

Substance added (10 μM)	Inhibition (%)
—	≡ 100
ATP	37 ± 5.9
ADP	40 ± 4.5
Adenylyl imido diphosphate	72 ± 2.7
5′-AMP	102 ± 2.7
3′-5′-AMP	101 ± 2.6
CTP	95 ± 2.4
GTP	98 ± 3.0
ITP	97 ± 2.7
UTP	99 ± 1.9
p-Nitrophenyl phosphate	98 ± 2.1
Sodium orthophosphate	98 ± 3.8

TABLE IV

EFFECT OF BUTANEDIONE ON Na^+-DEPENDENT PHOSPHORYLATION AND ($Na^+ + K^+$)-ATPase ACTIVITY

($Na^+ + K^+$)-ATPase (1.56 mg/ml) is incubated for 30 min at 25°C in 125 mM sodium borate buffer (pH 7.5) containing 5 mM $MgCl_2$ and 5 mM butanedione. Na^+-dependent phosphorylation and ($Na^+ + K^+$)-ATPase activity are determined as described under Materials and Methods and are compared with controls to which no butanedione is added. Results are given as means with S.E. of three experiments.

	Percentage of control
Na^+-dependent phosphorylation	21.8 ± 1.1
($Na^+ + K^+$)-ATPase	21.3 ± 1.2

apparent in all experiments. In 22 experiments under various conditions (pH, borate concentration, type and concentration of ligand, butanedione concentration), selected so as to give residual ($Na^+ + K^+$)-ATPase activities between 15 and 35% of the butanedione-free control, the residual K^+-stimulated *p*-nitrophenylphosphatase activity is 75% (S.E.: 2.2) of the control against an average resumal ($Na^+ + K^+$)-ATPase activity of 25% (S.E.: 1.2). The kinetics for the inactivation of *p*-nitrophenylphosphatase also differ from those for ($Na^+ + K^+$)-ATPase, viz. no pseudo first-order kinetics are found. These findings suggest

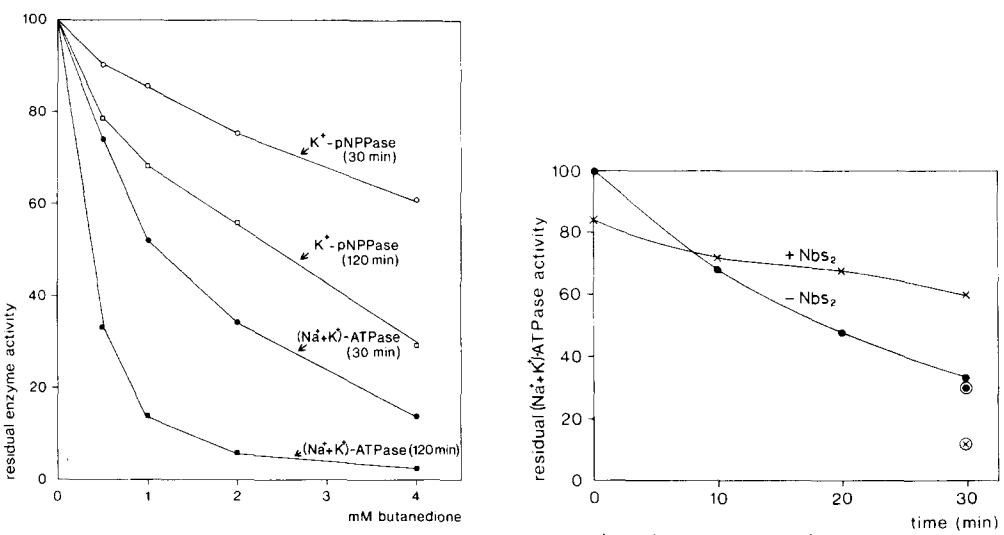

Fig. 6. Comparison between inactivation by butanedione of ($Na^+ + K^+$)-ATPase and K^+-stimulated *p*-nitrophenylphosphatase. ($Na^+ + K^+$)-ATPase (30 μg/ml) is preincubated for 120 min at 25°C with various concentrations butanedione in 125 mM borate buffer (pH 7.5), containing 5 mM Mg^{2+}. After 30 and 120 min ($Na^+ + K^+$)-ATPase and K^+-stimulated *p*-nitrophenylphosphatase (K^+-pNPPase) activities are determined as described under Materials and Methods. Enzyme activities are expressed as percent of control activity without butanedione.

Fig. 7. Effect of prior treatment with Nbs_2 on inactivation by butanedione. To 600 μl ($Na^+ + K^+$)-ATPase (100 μg/ml) in 125 mM borate buffer (pH 7.5), containing 5 mM $MgCl_2$, 30 μl of a 4 mM solution of Nbs_2 in 30 mM acetate buffer (pH 5.3) or buffer alone is added. After 1 h preincubation at 25°C butanedione is added (10 mM final concentration) and samples are taken at the indicated times. Dithioerythritol is added to a final concentration of 10 mM. To some samples (⊗, X, $+Nbs_2$; ◉, ●, $-Nbs_2$) no dithioerythritol is added. ($Na^+ + K^+$)-ATPase activity is determined as described under Materials and Methods. Enzyme activities are expressed as percent of control activity without butanedione.

that modification of more than one amino acid residue is necessary for complete inactivation of the *p*-nitrophenylphosphatase activity.

The *p*-nitrophenylphosphatase activity can be stimulated by ATP in the presence of Na^+, but only when K^+ is present in suboptimal concentration [28—30]. We have, therefore, investigated the effect of preincubation with butanedione in the presence of 0.5 mM K^+ with and without ATP. Table V shows that whereas the *p*-nitrophenylphosphatase activity in the absence of ATP at low K^+ concentration is not reduced by preincubation with butanedione, the stimulating effect of ATP is markedly reduced. This suggests that butanedione reacts with a residue which is involved in the stimulating effect of ATP.

Effect on sulfhydryl groups

Sulfhydryl residues seem to play an essential role in the active center of ($Na^+ + K^+$)-ATPase. Notwithstanding earlier findings that butanedione does not affect sulfhydryl groups [16,18], we have investigated whether the observed effects of butanedione could be due to interference with these groups. The number of reactive sulfhydryl groups before and after treatment with butanedione has been measured by absorbance spectrophotometry at 412 nm after reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) [24]. In both cases the number of titratable sulfhydryl groups was between 9 and 10, suggesting that butanedione does not react with sulfhydryl residues under the conditions of our experiments. However, since the reaction with butanedione is reversible and the one with Nbs_2 is irreversible this does not provide absolute proof for the above suggestion.

Next we have studied the interaction of the effects of Nbs_2 and butanedione.

TABLE V

EFFECT OF BUTANEDIONE ON *p*-NITROPHENYLPHOSPHATASE ACTIVITY MEASURED IN THE PRESENCE OF VARIOUS LIGANDS

($Na^+ + K^+$)-ATPase (31 μg/ml) is preincubated for 30 min at 25°C in 125 mM borate buffer (pH 7.5) containing 5 mM $MgCl_2$ with and without 2 mM butanedione. The *p*-nitrophenylphosphatase and ($Na^+ + K^+$)-ATPase activities are determined as described under Materials and Methods with the exception that in medium P the K^+, and Na^+ and ATP concentrations are as indicated below, while medium Q contains no K^+, but 10^{-4} M ouabain. Results are expressed as percent of the control measured under optimal conditions for *p*-nitrophenylphosphatase activity, and are given as averages with S.E. for four experiments.

Ligands present in assay	*p*-Nitrophenylphosphatase		
	— Butanedione	+ Butanedione	Ratio
1. Normal (5 mM K^+)	100	67 ± 10.1	0.67 ± 0.10
2. 0.5 mM K^+, 20 mM Na^+	22 ± 1.4	20 ± 2.9	0.91 ± 0.077
3. 0.5 mM K^+, 20 mM Na^+, 0.1 mM ATP	55 ± 3.6	29 ± 2.9	0.54 ± 0.058
4. Difference 3 — 2	34 ± 2.7	10 ± 2.6	0.30 ± 0.089
Ligands present	**($Na^+ + K^+$)-ATPase**		
	— Butanedione	+ Butanedione	Ratio
5. Normal (5 mM K^+, 55 mM Na^+)	100	19 ± 2.5	0.19 ± 0.025

For this purpose we have made use of the fact that inactivation by butanedione in Tris buffer can be reversed by gel filtration, whereas inactivation by Nbs_2 can be reversed by the addition of excess dithioerythritol. If butanedione and Nbs_2 do indeed react with different groups on the enzyme, inactivation by one reagent should not influence the effect of the other.

Fig. 7 shows the time dependence of the reaction of butanedione with $(Na^+ + K^+)$-ATPase, previously treated with Nbs_2, compared to that of an untreated control. After reaction with butanedione the samples are treated with dithioerythritol before assay of the $(Na^+ + K^+)$-ATPase activity. The difference at $t = 0$ is due to incomplete regeneration of the enzyme activity by dithioerythritol. The rate of inactivation by butanedione is clearly decreased after pretreatment with Nbs_2. Omission of dithioerythritol has no effect when butanedione is applied without pretreatment with Nbs_2, again suggesting that butanedione does not react with sulfhydryl groups. In a further experiment we have tested the effect of various combinations of butanedione, Nbs_2, dithioerythritol and gel filtration on the $(Na^+ + K^+)$-ATPase activity. Table VI shows that dithioerythritol is only able to reverse the effect of Nbs_2, while gel filtration only reverses (partially) the effect of butanedione. When both Nbs_2 and butanedione are used, the inactivation can only be completely reversed when both dithioerythritol and gel filtration are applied.

TABLE VI

INTERACTION OF Nbs_2 AND BUTANEDIONE ON $(Na^+ + K^+)$-ATPase ACTIVITY

To 190 μl of a $(Na^+ + K^+)$-ATPase suspension (1 mg/ml) in 125 mM sodium borate buffer (pH 7.5), containing 5 mM Mg^{2+}, 10 μl of a 4 mM solution of Nbs_2 in 30 mM acetate buffer (pH 5.3) or buffer alone is added. The mixture is incubated for 1 h at 25°C. Then 90-μl aliquots are mixed with 15 μl 11.3 mM butanedione or H_2O and incubated for 30 min at 25°C. Of these preparations 10-μl samples are diluted with 190 μl H_2O and 100-μl samples of these dilutions are added to 10 μl of either 100 mM dithioerythritol or H_2O and are kept at room temperature for 1 h. Other samples of the Nbs_2- and butanedione treated preparations and their controls are filtered through small (10 × 0.5 cm) Sephadex G-25 columns, or are first treated with dithioerythriol for 1 h followed by gel filtration with 50 mM Tris · HCl. After gel filtration the samples are kept at room temperature for 3 h, whereupon $(Na^+ + K^+)$-ATPase determinations are carried out as described under Materials and Methods. Results are expressed as percent of the appropriate control.

Treatment				Percent activity
Nbs_2	Butanedione	Dithioerythritol	Sephadex G-25	
—	—	—	—	≡ 100
—	+	—	—	27
+	—	—	—	29
+	+	—	—	13
—	—	+	—	110
—	+	+	—	31
+	—	+	—	97
+	+	+	—	51
—	—	—	+	≡ 100
—	+	—	+	51
+	—	—	+	24
+	+	—	+	37
—	—	+	+	≡ 100
—	+	+	+	58
+	—	+	+	103
+	+	+	+	89

These experiments strongly suggest that butanedione does not react with sulfhydryl groups in our experiments. The slower reaction with butanedione after pretreatment with Nbs_2 is probably due to steric hindrance by the large Nbs_2 residue or by conformational change induced by Nbs_2.

Amino acid analysis

Amino acid analysis after 120 min incubation with 4 mM butanedione does not show a significant decrease in any of the amino acids. Only when excess butanedione is not removed before acid hydrolysis there is a 90% reduction in the arginine content, but this is apparently due to a reaction of butanedione with arginine during acid hydrolysis at 110°C.

Assuming that $(Na^+ + K^+)$-ATPase has the stucture $\alpha_2\beta_2$ [31] and a molecular weight of 250 000 [32], the enzyme would contain 80—100 arginine residues per molecule [33,34]. Since amino acid analysis cannot significantly show a decrease of less than 5% in the arginine content, this means that less than five arginine residues can have been modified.

Determination of the number of free amino groups by the fluorescamine method [25] indicates that after butanedione treatment, leading to 88% inactivation, this number is 99.3% (S.E.: 1.0; $n = 4$) of the control without butanedione. This indicates that there is no significant modification of lysine or lipid amino residues, in agreement with the findings for the lysine content by amino acid analysis.

Discussion

This study clearly indicates that $(Na^+ + K^+)$-ATPase can be reversibly inactivated by butanedione. This is most likely due to modification of an arginine residue since it has been shown that this reagent is rather specific. The pH dependence of the reaction (Fig. 3), the dependence on the borate buffer concentration (Fig. 4) and the reversibility of the reaction after gel filtration with all buffers except borate (Table II) strongly support this conclusion.

The fact that the reaction obeys second-order kinetics indicates that a single amino acid residue is involved in the inactivating effect of butanedione, although modification of additional non-essential amino acid residues cannot be excluded. Our inability to find a significant decrease in arginine content by amino acid analysis also indicates that only few (less than five) arginine residues are modified. In several other cases this has been found, e.g. modification of 2 out of 161 arginine residues in beef heart mitochondrial ATPase leads to 50% inactivation [22]. It is unclear why in particular those residues which are important for enzyme activity are the ones that react primarily with butanedione. Powers and Riordan [21] suggest that residues located in a hydrophobic area would react primarily. It seems to us that such residues would have a lower apparent pK_a value, thus rendering them more reactive towards butanedione. This would also offer an alternative explanation for the pH dependence of the reaction, other than the effects on the borate buffer or another functional group nearby [10,16].

The fact that in many enzyme modification studies the inactivation reaction can be blocked by the addition of substrates or cofactors makes these reagents

valuable tools for the elucidation of the structure of the active site. The inactivation of $(Na^+ + K^+)$-ATPase can be blocked by ATP, particularly in the presence of CDTA (Fig. 5), which prevents the formation of a phosphorylated intermediate. Under these circumstances 2.5 μM ATP lowers inactivation by 50%; this concentration is only a factor 10 higher than the dissociation constant for ATP [35,36]. On the other hand, this concentration is much lower than the ATP concentration necessary to reduce the inactivating effects of *N*-ethylmaleimide (35 μM, Schoot et al., to be published) and of the disulfide of thioinosine triphosphate (100 μM, ref. 37). Out of a number of other phosphate compounds only ADP and the ATP analogue adenylyl imido diphosphate, which have affinities of the same order of magnitude for the enzyme [35,36], can reduce inactivation by butanedione in a concentration of 10 μM. The other phosphate compounds, which have much higher dissociation constants, have no effect at this concentration. The results strongly suggest that the arginine residue, which can be blocked by butanedione, is involved in the binding of nucleotides to the enzyme, possibly by binding of the negatively charged part of the ATP residue to the positively charged arginine residue.

An alternative explanation could be that binding of ATP would induce a change in enzyme conformation, which leads to burying of an essential arginine residue outside the active centre. This alternative seems unlikely to us, since in that case phosphorylation would re-expose this residue. Moreover, the parallelism between our findings and the fact that arginine is involved in the binding of negatively charged substrate or cofactor in a large number of other enzymes [10—22] seems more than accidental to us.

The much smaller protection of ATP in the presence of Mg^{2+} is probably the result of two effects. First, Mg^{2+}, in the presence of Na^+, stimulate phosphorylation by ATP, leading to removal of ATP from its binding site. Secondly, the presence of Mg^{2+} reduces nucleotide binding to the enzyme [38]. The latter mechanism explains the difference in protecting effect of ADP in the presence or absence of Mg^{2+}. Protection by ATP in the presence of Mg^{2+} is probably also influenced by the formation of ADP, which can also bind to the nucleotide binding site.

The reduced inactivation upon partial replacement of Na^+ by K^+ seems to be specific for $(Na^+ + K^+)$-ATPase, since it has not been found when other enzymes are inactivated by butanedione. This suggests that the presence of Na^+ in the preincubation medium makes the specific arginine residue more accessible to butanedione, probably due to a change in conformation of the enzyme. A 50% reduction of inactivation is observed at a Na^+/K^+ ratio of 30, suggesting that a K^+-sensitive site is involved in this effect. Nørby and Jensen [35] and Hegyvary and Post [36] have observed that binding of ATP to the enzyme is also diminished by K^+. This may mean that a conformational change in the ATP binding site induced by K^+ leads to a reduction in the binding of ATP as well as to a reduction in the accessibility of the essential arginine residue towards butanedione.

Modification of sulfhydryl groups also leads to inactivation of the $(Na^+ + K^+)$-ATPase activity and this inactivation can also be prevented by the addition of ATP in the absence of Mg^{2+} [5—9]. Patzelt-Wenczler et al. [9,37] suggest that a sulfhydryl group in the ATP binding centre is involved in the binding of

the 6-amino group of ATP. Since binding of ATP to a positively charged arginine residue probably involves a negatively charged phosphate group these results are not necessarily in conflict with each other. On the contrary, they may well explain the fact that modification of sulfhydryl residues with Nbs_2 partly prevents the reaction with butanedione (Fig. 7, Table IV). However, other explanations, like an aspecific conformational change due to reaction with Nbs_2, may also apply.

The equal sensitivity of the Na^+-dependent phosphorylation and the (Na^+ + K^+)-ATPase activity towards butanedione can be explained by assuming that the modification with butanedione prevents binding of ATP to the enzyme and thus all reactions following this step, like the Na^+-dependent phosphorylation. The lower sensitivity of the K^+-stimulated *p*-nitrophenylphosphatase towards butanedione can be explained by assuming that the binding of *p*-nitrophenyl phosphate takes place on a site different from that for ATP. Several authors have suggested a "half of the site reactive mechanism" for the (Na^+ + K^+)-ATPase system [39,40], in which two subunits with two phosphorylation sites are involved. Robinson [41] suggests that two phosphorylation sites, one with a high and one with a low affinity towards ATP are present on the two subunits. The (Na^+ + K^+)-ATPase reaction mechanism at physiological substrate concentrations would require phosphorylation of both subunits, while the activity of the K^+-stimulated *p*-nitrophenylphosphatase would require phosphorylation of the low affinity site only. The different sensitivities of the (Na^+ + K^+)-ATPase and *p*-nitrophenylphosphatase activities to butanedione could then be explained by assuming that butanedione primarily prevents binding of ATP to the high affinity site and thus phosphorylation of this site. This would also explain why ATP stimulation of the *p*-nitrophenylphosphatase activity is inhibited, since phosphorylation of the high affinity site of the enzyme would be involved in this activation.

Our conclusion that an arginine residue plays a role in the binding of ATP to (Na^+ + K^+)-ATPase is in agreement with the findings for several other enzyme systems, viz. mitochondrial ATPase [22], creatine kinase [18], glutamine synthetase [21] and carbamoyl phosphate synthetase [21]. Moreover, Borders and Riordan [18] have preliminary evidence that butanedione inhibits four other kinases, in which an ATP binding site may be involved. Peculiar to (Na^+ + K^+)-ATPase is that it requires only 1/100th of the ATP concentration needed by the other enzymes to prevent inactivation by butanedione, indicating a much higher affinity for ATP of (Na^+ + K^+)-ATPase.

Acknowledgements

Our thanks are due to Dr. P.L. Jørgensen for advice in the purification of (Na^+ + K^+)-ATPase and to Dr. F.J.M. Daemen for advice on the use of butanedione for modification of arginine and for helpful discussions in the course of this study. Miss Marlies Versteeg kindly carried out the amino acid analyses.

References

1 Bonting, S.L. (1970) In Membranes and Ion Transport (Bittar, E.E., ed.), Vol. I, pp. 257—363, Wiley-Interscience, London

2 Lindenmayer, G.E. and Schwartz, A. (1975) Pharmacol. Rev. 27, 1—34
3 Jørgensen, P.L. (1975) Q. Rev. Biophys. 7, 239—274
4 Dahl, J.L. and Hokin, L.E. (1974) Annu. Rev. Biochem. 43, 327—356
5 Skou, J.C. and Hilberg, C. (1965) Biochim. Biophys. Acta 110, 359—369
6 Banerjee, S.P., Wong, S.M.E., Khanna, V.K. and Sen, A.K. (1972) Mol. Pharmacol. 8, 8—17
7 Banerjee, S.P., Wong, S.M.E. and Sen, A.K. (1972) Mol. Pharmacol. 8, 18—29
8 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 234—245
9 Patzelt-Wenczler, R., Pauls, H., Erdmann, E. and Schoner, W. (1975) Eur. J. Biochem. 53, 301—311
10 Riordan, J.F. (1973) Biochemistry 12, 3915—3923
11 Daemen, F.J.M. and Riordan, J.F. (1974) Biochemistry 13, 2865—2871
12 Riordan, J.F. and Scandierra, (1975) Biochem. Biophys. Res. Commun. 66, 417—424
13 Borders, Jr., C.L., Riordan, J.F. and Auld, D.S. (1975) Biochem. Biophys. Res. Commun. 66, 490—495
14 Werber, M.M., Moldovar, M. and Sokolovsky, M. (1975) Eur. J. Biochem. 53, 207—216
15 Berghäuser, J. (1975) Biochim. Biophys. Acta 397, 370—376
16 Lange, L.G., Riordan, J.F. and Vallee, B.L. (1974) Biochemistry 13, 4361—4370
17 Foster, M. and Harrison, J.H. (1974) Biochem. Biophys. Res. Commun. 58, 263—267
18 Borders, Jr., C.L. and Riordan, J.F. (1975) Biochemistry 14, 4699—4704
19 Bleile, D.M., Foster, M., Brady, J.W. and Harrison, J.H. (1975) J. Biol. Chem. 250, 6222—6227
20 Blumenthal, K.M. and Smith, E.L. (1975) J. Biol. Chem. 250, 6555—6559
21 Powers, S.G. and Riordan, J.F. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2616—2620
22 Marcus, F., Schuster, S.M. and Lardy, H.D. (1976) J. Biol. Chem. 251, 1775—1780
23 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36—52
24 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70—77
25 Böhlen, P., Stein, S., Dairmon, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213—220
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
27 Jørgensen, P.L. (1974) Methods Enzymol. XXXII B, 277—290
28 Izumai, F., Nagai, K. and Yoshida, H. (1966) J. Biochem. Tokyo 60, 533—537
29 Yoshida, H., Nagai, K., Ohashi, T. and Nakagawa, Y. (1969) J. Biochem. Tokyo 171, 178—185
30 Robinson, J.D. (1973) Biochim. Biophys. Acta 321, 662—670
31 Giotta, G.J. (1976) J. Biol. Chem. 251, 1247—1252
32 Kepner, G.R. and Macey, R.I. (1968) Biochem. Biophys. Res. Commun. 30, 582—587
33 Kyte, J. (1972) J. Biol. Chem. 246, 4157—4165
34 Perrone, J.R., Hackney, J.F., Dixon, J.F. and Hokin, L.E. (1975) J. Biol. Chem. 250, 4178—4184
35 Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104—116
36 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5234—5240
37 Patzelt-Wenczler, R. and Schoner, W. (1975) Biochim. Biophys. Acta 403, 538—543
38 Kanaiike, K., Erdmann, E. and Schoner, W. (1973) Biochim. Biophys. Acta 298, 901—905
39 Stein, W.D., Lieb, W.R., Karlish, S.J.D. and Eilam, Y. (1973) Proc. Natl. Acad. Sci. U.S. 70, 275—278
40 Repke, K.R.H. and Schon, R. (1973) Acta Biol. Med. Ger. 31, 19—30
41 Robinson, J.D. (1975) Ann. N.Y. Acad. Sci. 243, 60—72