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CHARACTERIZATION OF ESSENTIAL ARGINYL RESIDUES IN SHEEP KIDNEY $(Na^+ + K^+)$-ATPase

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SUMMARY

Treatment of highly purified sheep kidney medulla $(Na^+ + K^+)$-ATPase with 2,3-butanedione results in a rapid inactivation of the enzyme. Contrary to a previous report using rabbit kidney enzyme (DePont et al., Biochim. Biophys. Acta (1977) 482, 213), the inactivation is biphasic under a variety of experimental conditions, with a rapid, initial inactivation which is followed by a slower loss of activity. The second, slower phase of the inhibition obeys pseudo-first order kinetics, with a second order rate constant for inhibition of 20 $min^{-1}M^{-1}$. ATP and ADP provide no protection in the initial phase of the inhibition, but protect the enzyme completely from the second phase of the inhibition. AMP, while less effective than ATP and ADP, provides a partial protection of the enzyme activity from inhibition by 2,3-butanedione. Inorganic phosphate provides partial protection in both phases of the inactivation. Adenosine alone is without effect, but adenosine plus inorganic phosphate provides a greater protection than phosphate alone. The results indicate that either (1) two or more active site residues or (2) a single arginine, experiencing different reactivities in two different active site conformations, are essential to $(Na^+ + K^+)$-ATPase activity.

INTRODUCTION

A thorough understanding of membrane-bound $(Na^+ + K^+)$-ATPase and its role in monovalent cation transport in mammalian systems will require the characterization of the amino acid residues involved in the binding of substrates and metal ion activators to the enzyme. Recently DePont et al. (1) reported the inactivation of the $(Na^+ + K^+)$-ATPase from rabbit kidney by 2,3-butanedione, a reagent used widely for the detection of essential arginine residues in the active centers of enzymes (2-5). These workers

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describe simple second order inhibition kinetics and suggest that a single arginyl residue is essential to $(Na^+ + K^+)$-ATPase activity. Our own examination of the inhibition of the $(Na^+ + K^+)$-ATPase reveals a biphasic response to 2,3-butanedione. Our data suggest the involvement of two or more active site arginines or, alternatively, a single arginine which experiences different reactivities to butanedione in two different active site conformations.

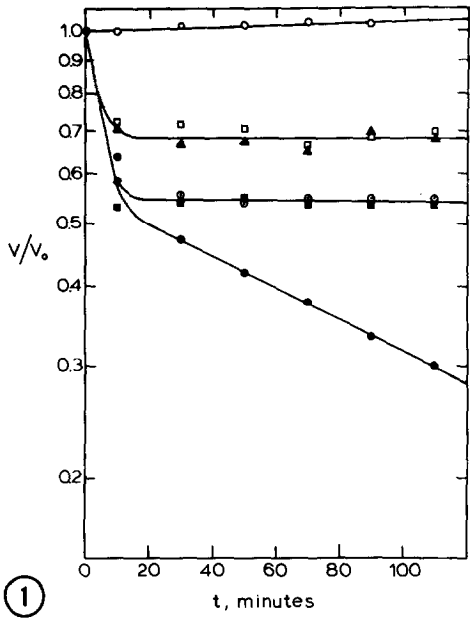
MATERIALS AND METHODS

The $(Na^+ + K^+)$-ATPase used in these studies was prepared from sheep kidney medulla as previously described (6) and was 90-95% pure as judged from SDS gel electrophoresis. 2,3-Butanedione was obtained from Aldrich, twice distilled (b.p. 87.2°C) and stored at -13°C until the day of use, when an 11.4 x 10^{-3} M solution in water was prepared.

Enzymatic activity was measured using a coupled assay as previously described (7). In this assay the hydrolysis of ATP was coupled to the oxidation of NADH using pyruvate kinase and lactic dehydrogenase. Except as noted the modification of the enzyme with 2,3-butanedione was carried out at 24°C, pH 7.4 in 0.04 M TES, 0.02 M borate, 0.1 mg/ml $(Na^+ + K^+)$-ATPase. The reaction was initiated by addition of 2,3-butanedione and stopped by a 100-fold dilution into the ATPase assay solution. Under the conditions used, the butanedione thus added to the assay mixture had no effect on the activities of the assay enzymes.

RESULTS AND DISCUSSION

The inhibition of the $(Na^+ + K^+)$-ATPase by 2,3-butanedione and the protection afforded by ATP, ADP and inorganic phosphate are shown in Figure 1. ATPase activity is completely abolished after long incubation times and the time course, especially at low butanedione concentrations,seems to be biphasic. A rapid initial inactivation is complete after 10-20 minutes and is followed by a much slower inhibition process. The rate of the initial inactivation process varied between preparations



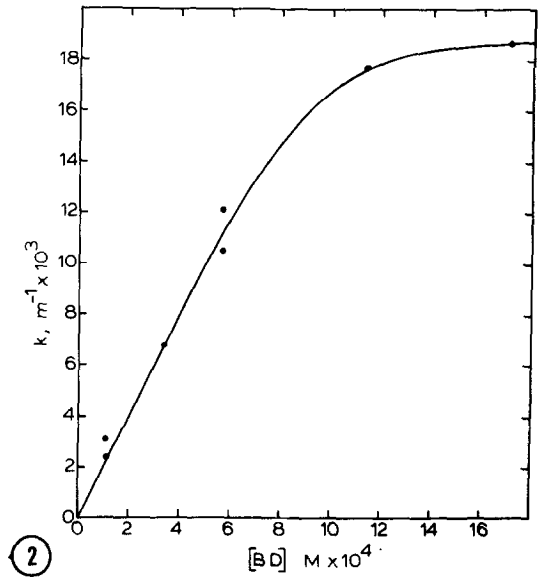


Figure 1. Inactivation of kidney medulla ($Na^+ + K^+$)-ATPase by 2,3-butanedione and protection by ATP, ADP and inorganic phosphate. The enzyme was treated with butanedione and assayed as described in the text. The incubation medium contained 0.228 mM butanedione only (●), or 0.228 mM butanedione plus 3 mM ATP (■,□), 3 mM ADP (⊙,▲) and/or 10 mM sodium phosphate (□,▲). A sample was also incubated without butanedione or added substrates (○). V_o is the initial enzyme activity, while V is the enzyme activity after incubation for t minutes.

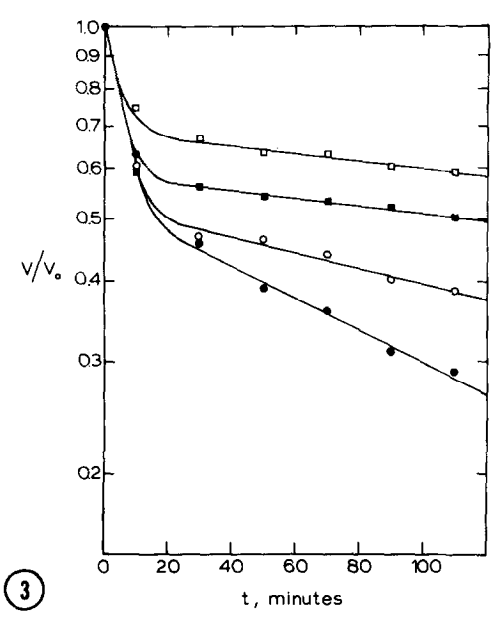
Figure 2. The apparent first order rate constants for inhibition of the ($Na^+ + K^+$)-ATPase as a function of 2,3-butanedione concentration.

of the enzyme. The observed rate constant for the second, slower process varies linearly with butanedione below 0.6 mM, but is essentially independent of butanedione concentration above 1.2 mM, as shown in Figure 2. As shown in Figure 1 the presence of 3 mM ATP or ADP in the butanedione incubation had no effect on the initial, rapid inactivation of the enzyme, but completely blocked the second, slower inhibition process. In these respects ADP proved to be fully as effective as ATP itself. When the incubation medium contained 10 mM sodium phosphate in addition

to ATP or ADP, a further protection against inhibition was consistently observed. Again in this case no difference between ATP and ADP was detected. The additional protection by phosphate appeared to occur in the initial, rapid phase of the enzyme-butanedione reaction and generally protected 30-35% of that part of the enzyme activity which was normally affected in the initial phase of the inhibition.

Since ATP and ADP acted identically in protecting the ATPase from butanedione, AMP and adenosine were examined in a series of similar experiments. As shown in Figure 3, 3×10^{-3} M AMP partially protected the enzyme from the second phase of the inhibition, but was not as effective as ATP and ADP in this respect. Increasing the AMP concentration by a factor of 10 provided no additional protection against inhibition, suggesting that AMP is simply less able to protect the enzyme from butanedione. Adenosine alone (Figure 4) had no effect on the inhibition reaction. An unusual effect was observed in a comparison of inorganic phosphate, adenosine and adenosine plus phosphate. As shown in Figures 3 and 4, sodium phosphate alone provides a partial protection against butanedione at levels of 1×10^{-2} M. However the combination of adenosine and inorganic phosphate in the butanedione incubation provides a substantial protection against inactivation which is not observed in the presence of either reagent alone. With 3×10^{-3} M adenosine and 1×10^{-2} M sodium phosphate the rate constant for the second phase of the inhibition was reduced by 48.5%.

The time course of butanedione inhibition and the specificity of the protection of the ATPase by substrates indicates that either (1) two or more arginines or (2) a single arginine with different reactivities in two active site conformations may be essential to activity. From the linear portion of Figure 2, a second order



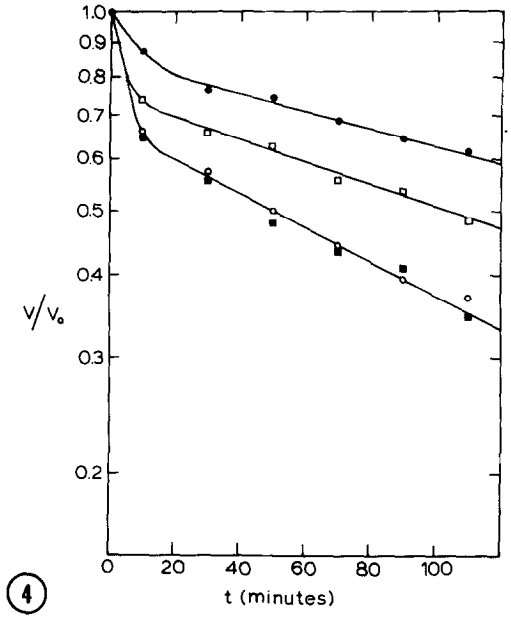
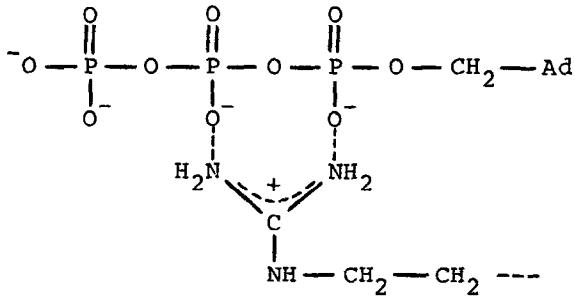


Figure 3. Protection against butanedione inhibition by AMP and inorganic phosphate. The incubation medium contained 0.228 mM butanedione only (●), or 0.228 mM butanedione plus 3 mM AMP (■,□), and/or 10 mM sodium phosphate (○,□). V_o, V and t are defined in Figure 1.

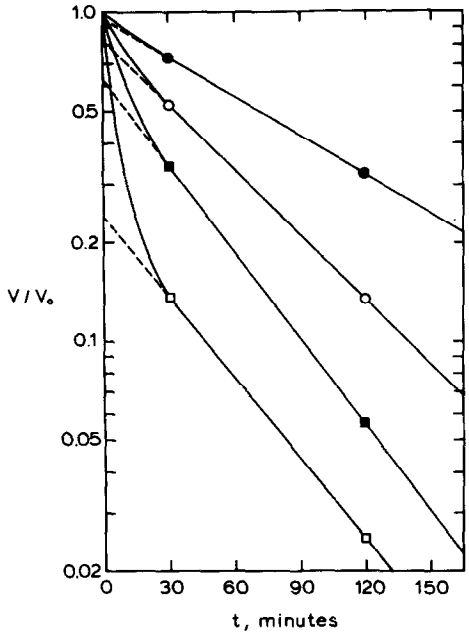
Figure 4. Effects of adenosine and inorganic phosphate on butanedione inhibition. The incubation medium contained 0.228 mM butanedione only (■), or 0.228 mM butanedione plus 3 mM adenosine (○,●) and/or 10 mM sodium phosphate (□,●). V_o,V and t are defined in Figure 1.

rate constant for inhibition of 20 $min^{-1}M^{-1}$ can be obtained for the second phase of the inhibition. This is similar to the value of 19 $min^{-1}M^{-1}$ measured with the rabbit kidney ($Na^+ + K^+$)-ATPase (1). The protection afforded by ATP, which completely eliminates the second phase of the butanedione inhibition, strongly suggests the presence of an arginyl residue at the ATP site of the enzyme. The identical protective effects of ATP and ADP strongly suggest that the terminal, or γ-, phosphate of ATP is not involved in protection of the suspect arginine. The partial protection observed with saturating concentrations of AMP compared with the complete protection

afforded by ADP could be explained if the arginyl group is only partially blocked with AMP at the nucleotide binding site, but is totally inaccessible when ADP is present at this site. These data could be explained if the arginyl residue in question bridged the α- and β- phosphates of ATP at the ATP binding site, as shown below.



The reasons for the differences between our data and those reported for rabbit kidney (1) are not obvious. When sheep kidney enzyme is treated with butanedione under the conditions used with the rabbit enzyme (125 mM sodium borate, 5 mM $MgCl_2$, pH 7.5 (1)), biphasic inhibition kinetics are still observed. It is interesting in this regard that Figure 6 of DePont et al. presents evidence of biphasic inhibition kinetics. When these data are carefully replotted (our Figure 5), biphasic plots are obtained at each concentration of butanedione used. The replotted data are generally more consistent with the biphasic data presented here than with the linear kinetics of Figure 1 of DePont et al. Rate constants calculated from the replotted data of DePont et al. in Figure 5 are compared with our own data at the same concentrations of butanedione in Table I. The agreement between these data is remarkably good. It would appear that the data of Figure 1 in DePont et al. (1) presents an incomplete picture of the inhibition of the $(Na^+ + K^+)$-ATPase by 2,3-butanedione.



igure 5. Replotted data of Figure 6 from reference 1 for inhibition of rabbit kidney ($Na^+ + K^+$)-ATPase by butanedione. Conditions of the incubation were: 125 mM sodium borate, 5 mM $MgCl_2$, pH 7.5 and 0.5 mM (●), 1 mM (○), 2 mM (■) or 4 mM (□) 2,3-butanedione. V_o, V and t are defined in Figure 1.

TABLE I

COMPARISON OF OBSERVED RATE CONSTANTS FOR INHIBITION BY BUTANEDIONE OF SHEEP KIDNEY AND RABBIT KIDNEY ($Na^+ + K^+$)-ATPase

Butanedione Concentration	Apparent First Order Rate Constant for Inhibition, k_{app}*	
	Present Work (Sheep Kidney)	Data of DePont et al. (1) (Rabbit Kidney)
0.5 mM	9.8×10^{-3} min^{-1}	9.0×10^{-3} min^{-1}
1.0 mM	16.7	14.9
2.0 mM	19.0	19.8
4.0 mM	19.0	18.7

*In both cases the value of k_{app} was calculated from the linear portion of the second phase of the inhibition curve.

Our data, as well as those of DePont et. al. (our Figure 5), are not consistent with a single arginine residue existing in a single conformational state. A possible explanation would involve two arginyl residues at the active site, one which reacts rapidly with 2,3-butanedione and one which reacts slowly. Alternative explanations can be found in models involving a single arginine and either slowly interconverting forms of the active site or alternating site cooperativity of the type described by Hackney and Boyer (8). In the former case, a single essential arginine could react rapidly with butanedione in one conformation or slowly in a second state. Then the second phase of the inhibition time course could be reflecting either the rate of the slowly reacting arginine or the rate of interconversion of the enzyme, whichever is faster. In the latter model, substrate binding at one site promotes catalytic steps of bound reactants at another site. If the regulatory site, for example, possesses a rapidly reacting arginine and the catalytic site a more slowly reacting residue, then partial inhibition might result when the more available arginine reacts. If some enzyme activity persists in the absence of site-site cooperativity, then the reaction of 2,3-butanedione with one residue per site would be necessary and sufficient for complete inhibition.

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