INHIBITION OF $(Na^+ + K^+)$-ACTIVATED ATPase BY *N,N'*-DICYCLOHEXYLCARBODIIMIDE

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1. Introduction

Hydrolysis of ATP by a membrane-bound $(Na^+ + K^+)$-activated ATPase is assumed to proceed via the following reactions [1] (eqs. (1) and (2)):

$$ATP + E \overset{Mg^{2+},Na^+}{\rightleftharpoons} E \sim P + ADP \qquad (1)$$

$$E \sim P + H_2O \overset{K^+}{\longrightarrow} E + P_i \qquad (2)$$

Incubation of the protein-bound phosphate obtained by acid precipitation of the enzyme with NH_2OH results in the release of inorganic phosphate [2–4]. This finding points to an acyl phosphate. In the native enzyme, however, so far an intermediary formation of an acyl phosphate could not be demonstrated: Incubation of the native enzyme with ATP, Na^+ and NH_2OH which should be expected to result in a chemical alteration of the phosphate acceptor group by the formation of hydroxamic acids, did not inactivate the enzyme [5,6]. This failure could either be due to a transformation of an acid labile phosphate into an acid stable product during the procedure of acid denaturation [7], or to a protection of the phosphorylated intermediate by ATP and Na^+ against the nucleophilic attack by NH_2OH.

In order to evaluate a possible role of carboxylic acid groups in the active center of $(Na^+ + K^+)$-activated ATPase, assays were performed with *N,N*-dicyclohexylcarbodiimide (DCCD) which is known to react with carboxylic acid groups [8]. This paper reports the protective effects of Na^+, K^+ and ATP against the inhibition of $(Na^+ + K^+)$-activated ATPase by DCCD. It is assumed that a carboxylic acid group is located in the active center of the enzyme.

2. Methods

$(Na^+ + K^+)$-activated ATPase from ox brain cortex was purified as described previously [9]. 1.7 to 2.2 mg enzyme protein (specific activity 1.4 to 3.9 U./mg protein) were incubated in a total volume of 1 ml at 10°C with 60 mM imidazole pH 7.2, 0.2 ml of 0.01 M DCCD in ethanol and the additions shown in the legends of the figures. Samples were withdrawn from the incubation mixture at various intervals and the enzymatic activity determined with the coupled optical assay [9] in an Eppendorf photometer equipped with an automatic sample changer. All measurements were corrected for a control containing no DCCD.

3. Results and discussion

Incubation of $(Na^+ + K^+)$-activated ATPase with 2 mM DCCD results in an instantaneous 25% inhibition of the enzymatic activity (fig. 1). This immediate response is followed by a second slow phase of enzyme inactivation depending on temperature (fig. 1). The activity of a Na^+ and K^+ independent ATPase, which is always present in the enzyme preparations, was not affected by this treatment (not shown). An instantaneous and a slow response on addition of this reagent was also found by Beechey et al. [10,11] in studies on the inhibition of oxidative phosphorylation. As may be seen from fig. 2, half maximal inhibition of the enzyme is observed at 9×10^{-4} M DCCD. This concentration is far above that necessary for half maximal inhibition of pyridine nucleotide transhydrogenase and ATP-linked reduction of succinate in mitochondria

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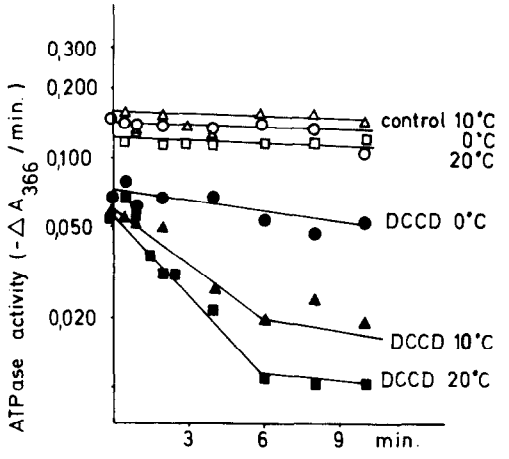

Fig. 1. (left curve). Effect of temperature on the inactivation of $(Na^+ + K^+)$-activated ATPase by DCCD, 2.58 mg ATPase was incubated for various intervals with 2 mM DCCD. At the time indicated aliquots were withdrawn from the incubation mixture and tested for enzymatic activity. Open symbols represent values in the absence of DCCD, closed symbols in the presence of 2 mM DCCD.

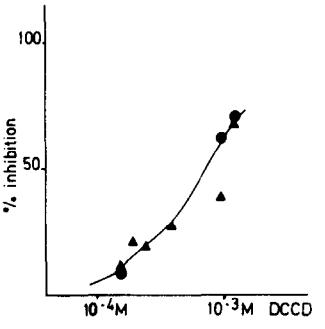

Fig. 2. (right curve). Effect of the concentration of DCCD on the inactivation of $(Na^+ + K^+)$-activated ATPase. Activity was determined after a 6 min incubation period. The amount of ethanol was kept constant. Triangles and circles represent separate experiments.

[10] and of a membrane-bound ATPase in *Streptococcus faecalis* [12].

Under the assumption that DCCD acts on part(s) of the active center of the enzyme, a possible protective effect of the substrate ATP or the activators Na^+ and K^+ against the inactivation by DCCD was tested. As shown in fig. 3A 4 mM ATP completely inhibited the inactivation of the enzyme by DCCD. Even both activators, Na^+ or K^+ at 60 mM, almost totally

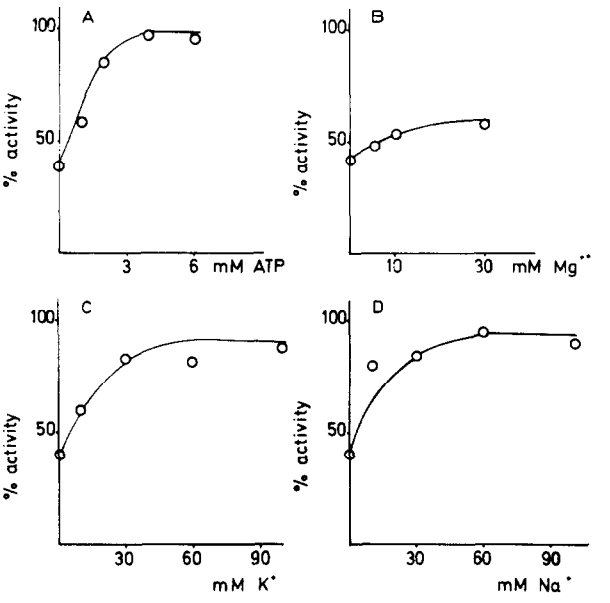

Fig. 3. Effects of ATP and various cations on the activity of $(NA^+ + K^+)$-activated ATPase in presence of 2 mM DCCD. The enzyme was preincubated for 6 min. (A) Correlation between ATP concentration and enzymatic activity. (B) Correlation between Mg^{2+} concentration and enzymatic activity. (C) Correlation between K^+-concentration and enzymatic activity. (D) Correlation between Na^+-concentrations and enzymatic activity.

counteracted the inactivating effect of this reagent (fig. 3C,D). The identical correlations between the cation concentrations and the extent of protecting the enzymatic activity (fig. 3C,D) indicate an action of both cations at the same site. Mg^{2+} ions which are necessary for the hydrolysis of ATP in the overall reaction (eqs. (1), (2)) were almost without any effect on the inactivation by DCCD (figs. 3B, 4). As may be seen from a comparison of figs. 3A and 4, however, Mg^{2+} ions at 6 mM considerably counteracted the protective effect of ATP; although ATP alone (4 mM) could abolish the inhibitory action of DCCD completely, the same concentration of ATP in presence of 6 mM Mg^{2+} prevented the DCCD-caused inactivation by only 25%. This may be due to a complex formation of Mg^{2+} with ATP. However, a combination of ATP, Na^+ and K^+ in concentrations generally used for assaying the activity of $(Na^+ + K^+)$-activated ATPase

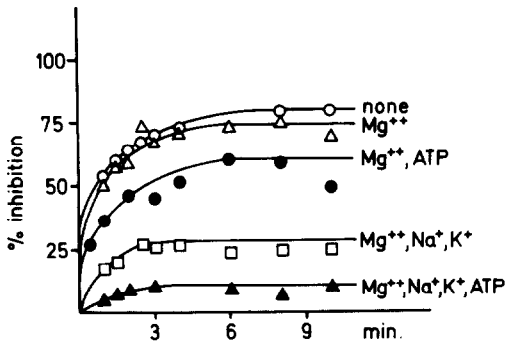

Fig. 4. Kinetics of the effects of ATP and various cations on the inhibition of $(Na^+ + K^+)$-activated ATPase by 2 mM DCCD. ○ = no additions; △ = 6 mM $MgCl_2$; ● = 6 mM $MgCl_2$, 4 mM ATP; □ = 6 mM $MgCl_2$, 100 mM NaCl, 10^2 mM KCl; ▲ = 4 mM ATP, 6 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl.

overcame the counteracting effect of 6 mM Mg^{2+} (fig. 4).

These findings show that DCCD acts in a specific way on the active site of $(Na^+ + K^+)$-activated ATPase. Mg^{2+} ions may counteract the protective effects of ATP, Na^+ and K^+ against the DCCD-caused inactivation either by lowering the affinity of these substrates to the enzyme or by a conformation change of the enzyme. A more detailed kinetic investigation should clarify this question.

Carbodiimides are known to react with carboxylic acid groups [8]. In accordance with results obtained from the acid denatured phosphorylated enzyme it may be suggested that a carboxylic acid group is the phosphate acceptor group in the native enzyme. In former experiments the negative results in demonstrating an inactivation of the enzyme by NH_2OH [5,6] were most probably due to a protective effect of ATP and Na^+ on the phosphorylated acceptor group against the nucleophilic attack of NH_2OH. A hydrolysis of the protein-bound phosphate by NH_2OH in the native enzyme observed earlier [5] could be traced down to small amounts of NH_4^+ formed by decomposition of NH_2OH. NH_4^+ ions may replace K^+ ions in eq. (2). Studies on the site of action of DCCD with the radioactive compound are in progress.

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