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On the site of action of *N,N'*-dicyclohexylcarbodiimide as an inhibitor of $(Na^+ + K^+)$-activated ATPase*

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N,N'-D CYCLOHEXYLCARBODIIMIDE, (DCCD) inhibits the membrane-bound $(Na^+ + K^+)$-activated ATPase[1,2] from ox brain. This enzyme catalyses the active transport of Na^+ and K^+-ions through cellular membranes.[3] DCCD shows in mitochondria oligomycin-like properties[4] by reacting covalently either with an intermediate in oxidative phosphorylation or with a membrane component essential for the functioning of an intermediate.[5] Because both reagents, DCCD and oligomycin, are inhibitors of $(Na^+ + K^+)$-activated ATPase[1,2,6] we studied if DCCD shows oligomycin-like actions also on transport ATPase.

We found that DCCD in contrast to oligomycin inhibits the Na^+-dependent phosphorylation reaction. The sites reacting with DCCD are protected against the attack of this substance by Na^+ but not K^+ under conditions, when a phosphorylated intermediate is formed. The sites protected by Na^+ could be labeled and partially purified as the radioactive derivatives.

METHODS AND MATERIALS

$(Na^+ + K^+)$-activated ATPase from ox brain cortex was purified and assayed as described earlier.[7] One mg enzyme protein hydrolysing 1–3 μmoles ATP/min × mg protein was incubated at 10° in a total volume of 1 ml containing 0·2 ml ethanol, 60 μmoles imidazole pH 7·25 and the additions mentioned in Fig. 1. DCCD (1 μmole) was dissolved in 0·2 ml ethanol. Samples of 0·03 ml were

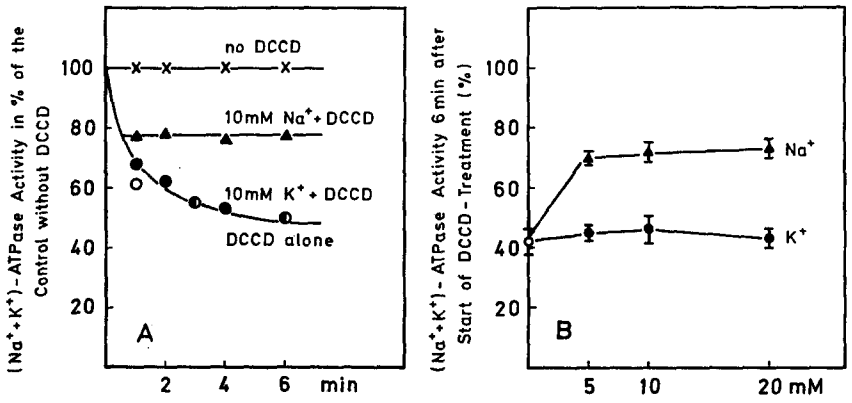

FIG. 1.(A) Kinetics of inactivation of $(Na^+ + K^+)$-activated ATPase by DCCD: Incubation at 10° in the absence of DCCD (×); with DCCD (○); with DCCD, 0·5 mM ATP, 0·5 mM $MgCl_2$, 10 mM K (●) and with DCCD, 0·5 mM ATP, 0·5,mM $MgCl_2$, 10 mM Na^+ (▲). (B) Effects of the concentrations of Na^+ or K^+ in the presence of 0·5 mM $MgCl_2$ + 0·5 mM ATP on $(Na^+ + K^+)$-ATPase activity after a 6 min incubation with DCCD. The values are means ± S.E.M. of four experiments. Student's *t*-test for statistical significance. $P < 0{\cdot}001$.

withdrawn from the incubation mixture at various intervals and the enzymatic activity was determined at 37°. No further inactivation occurred under the conditions of the coupled optical assay,[7] as was evident from the linear decrease of NADH. In order to exclude any inhibition of the enzyme by ethanol[8] all experiments were corrected for a blank containing all ingredients, but no DCCD.

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Phosphorylation experiments were performed according to Post *et al.*[9] ^{14}C-methylamine (spec. act. 54 mc/mmole) and (^{32}P)-γ-ATP were purchased from the Radiochemical Centre, Amersham, England. ^{3}H-methylamine (34·16 mc/mmole) from NEN Chemicals, Dreieichenhain, Germany. Ethyl-(3-(dimethylamino)-propyl)-carbodiimide from Schuchardt GmbH, München; all other reagents were from Merck, Darmstadt and Boehringer, Mannheim.

RESULTS AND DISCUSSION

Incubation of ($Na^+ + K^+$)-activated ATPase with DCCD results in an immediate inhibition of 20 per cent followed by a time-dependent decrease of enzymatic activity (Fig. 1A). Na^+-ions but not K^+-ions diminished the time-dependent inactivation at concentrations as low as 5 mM (Fig. 1B). No protection against the inactivation by DCCD was seen, when K^+ ions were substituted by Rb^+ and Cs^+. In contrast to these findings, alkali ions protect the enzyme against the inactivation in the absence of ATP and Mg^{2+} (Table 1). The disappearance of the protective effects of K^+, Rb^+ and Cs^+ is found only in the presence of ATP and Mg^{2+}; when ATP was replaced by ITP or inorganic phosphate, or when Mg^{2+} was omitted; all alkali ions with the exception of Li^+ protected the enzyme against the inactivation. The ATP-concentration of 0·5 mM, used in Fig. 1, does not influence the inactivation by DCCD. Thus a "Na^+"-specific effect is seen only under conditions when a phosphorylated intermediate is formed. A comparison of the data from Fig. 1B and Table 1 support the interpretation that Mg^+ + ATP induce a change of the enzyme conformation in the presence of K^+, Rb^+ and Cs^+. Effects similar to those reported here were seen with the hydrophobic carbodiimide diphenylcarbodiimide but not with water-soluble carbodiimides or dibromacetophenone. The latter reagent modifies carboxyl groups of proteins like carbodiimides.[10,11]

TABLE 1. EFFECTS OF 60 mM UNIVALENT CATIONS ON THE INACTIVATION BY DCCD

Cation	ATPase-activity in % of the respective control without DCCD	*p*-value of Student's *t*-test
None	38·5 ± 4·1 (6)	
Li^+	42·3 ± 2·5 (6)	>0·1
Na^+	73·6 ± 2·1 (5)	<0·001
K^+	69·8 ± 1·0 (5)	<0·001
Rb^+	66·4 ± 2·6 (5)	<0·001
Cs^+	62·8 ± 3·8 (5)	<0·001

Incubation for 6 min at 10° with 1 μmole DCCD/mg protein and 60 mM imidazole pH 6·4. ATPase-activity in percentage of the control containing no DCCD ±S.E.M. Number of experiments in parentheses.

ATP-hydrolysis by ($Na^+ + K^+$)-activated ATPase proceeds via a Na^+-dependent phosphorylation of the membrane protein followed by a K^+-dependent hydrolysis of the intermediate.[9] In order to localize the action of DCCD in this reaction sequence, the inactivation-kinetics of the overall-reaction of ($Na^+ + K^+$)-activated ATPase and the Na^+-dependent incorporation of radioactive phosphate into the enzyme protein from γ[^{32}P]-ATP, were compared (Fig. 2). Until 3 min after onset of the inactivation, the overall reaction was more affected than the phosphorylation reaction. This finding indicates that DCCD acts on Na^+-dependent phosphorylation and a subsequent reaction. DCCD which shows in mitochondria oligomycin-like properties[4] thus differs in ($Na^+ + K^+$)-activated ATPase in its site of action from oligomycin. Oligomycin does not affect Na^+-dependent phosphorylation but inhibits a subsequent reaction.[12]

The inhibitory action of DCCD on the phosphorylation reaction (Fig. 2) could be interpreted to be due to a reaction with the phosphate acceptor group. But this conclusion is not justified, since univalent cations protect the enzyme also in the absence of ATP (Table 1). The site reacting with the DCCD thus appears to be remote from the phosphate acceptor group. During the inactivation of ($Na^+ + K^+$)-activated ATPase by DCCD most probably covalent bonds are formed. This is concluded from the finding that the inhibitory effect of DCCD cannot be reversed by intensive washing of the enzyme and also from isotope double-labelling studies (Table 2): The part of the enzyme

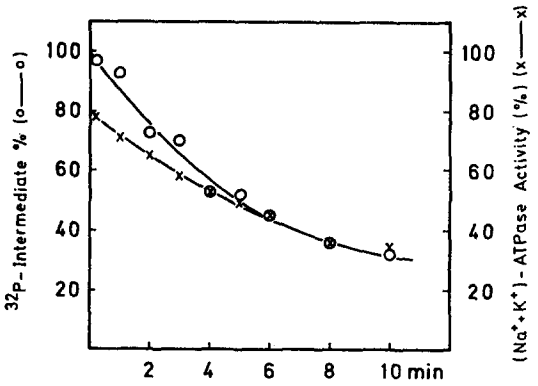

FIG. 2. Kinetic of the action of DCCD on Na^+-dependent phosphorylation reaction and ($Na^+ + K^+$)-ATPase activity.

which is protected against the attack of DCCD in the presence of ($Na^+ + Mg^{2+} + ATP$) but not of ($K^+ + Mg^{2+} + ATP$) seems to be essential for the catalytic function. Since labelling of this site could give information on the enzyme structure, the enzyme pretreated with DCCD in the presence of ($Na^+ + Mg^{2+} + ATP$) was transformed to the ^{14}C-methylamine derivative by a second incubation with ethyl(3-(dimethylamino)-propyl)-carbodiimide. The ^3H-methylamine derivative of the enzyme pretreated with DCCD and ($K^+ + Mg^{2+} + ATP$) was obtained by the same way. A water soluble

TABLE 2. DOUBLE ISOTOPE LABELLING OF DCCD-PRETREATED ($Na^+ + K^+$)-ATPASE

Treatment	($Na^+ + K^+$)-ATPase activity (μmoles/min/mg) DCCD + (Na^+, Mg^{2+}, ATP)	DCCD + (K^+, Mg^{2+}, ATP)	
Before addition of DCCD	4·56	4·56	
After 6 min incubation	3·7	2·9	
After washing	2·4	1·8	
	^{14}C-methylamine incorporated (dis./min/fraction)	^3H-methylamine incorporated (dis./min/fraction)	$^{14}C/^3H$
Incubation for 12 hr with methylamine + carbodiimide; mixing of both fractions	8×10^5	6×10^5	1·3
Combined supernatants after extraction with 10% Triton X-100	285,450	76,700	3·8
Separation by electrofocusing			
Fraction 7, pH 1·6	2780	500	5·5
Fraction 50, pH 8·2	1296	250	5·2

9·45 mg ATPase protein was incubated in Corex-glass tubes in a total volume of 16 ml at 10° for 6 min with 10·9 μmoles DCCD dissolved in 1 ml ethanol, 8 μmoles ATP, 8 μmoles $MgCl_2$ and 320 μmoles NaCl or KCl. After two washings with 1 mM dimercaptopropanol containing 1 mM ATP the membranes were incubated in 60 mM imidazole pH 6·3, 100 mM ethyl-(3-(dimethylamino)-propyl)-carbodiimide and 28 mM radioactive methylamine (ca. 10^6 dis./min/μmole) at 20° for 12 hr (16 ml total vol.). The membranes were washed thereafter four times with 30 ml 1 M methylamine. ^3H and ^{14}C labelled membranes were mixed and extracted with 5 ml 10% Triton X-100 (three times). The combined supernatants were further separated by electrofocusing at 500 V for 3 days in a 110 ml column.

carbodiimide was used as a coupling agent during the second incubation because this carbodiimide in contrast to hydrophobic carbodiimides has been shown to modify quantitatively carboxyl groups in proteins.[11] After mixing of the ^{14}C- and ^{3}H-methylaminated membrane derivatives and fractionation of the membranes all groups should be doubly labelled except those which had been protected against the attack of DCCD by the presence of ($Na^+ + Mg^{2+} + ATP$). The groups protected by ($Na^+ + Mg^{2+} + ATP$) should be recognized by an increase in the $^{14}C/^{3}H$ ratio after fractionation of the double labelled mixture. Table 2 shows that the membrane components which are protected in the presence of ($Na^+ + Mg^{2+} + ATP$) against the attack by DCCD can be solubilized by treatment with Triton X-100. Isoelectric focusing of this extract gives two radioactive peaks with isoelectric points at pH 1·6 and 8·2 and a $^{14}C/^{3}H$-ratio above 5. The demonstration of two different labelled membrane components supports the interpretation of Fig. 2 that DCCD acts on at least two different sites. The data of Table 2 also indicate that the ($Na^+ + K^+$)-activated ATPase consists of at least two components with different charges. This finding supports the assumption that ($Na^+ + K^+$)-activated ATPase is a multienzyme system.[13]

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