INHIBITORY ACTION OF CARBODIIMIDES ON BACTERIAL MEMBRANE ATPASE.[1]

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SUMMARY. Dicyclohexylcarbodiimide (DCCD) inhibits the membrane ATPase of Streptococcus fecalis indirectly through a reaction with another membrane component as yet unidentified. Whether or not DCCD reacts covalently to produce this inhibition is also not known. We have now found that several other carbodiimide compounds differing greatly from DCCD in structure and water solubility are also inhibitory. However, the hydrophobic carbodiimides were more potent by many orders of magnitude. Since structurally diverse carbodiimides could inhibit the membrane-bound ATPase we conclude that the inhibition results from a covalent reaction. The relatively high potency of the hydrophobic carbodiimides indicates that the reactive site is located within a non-polar region of the membrane. It is suggested that hydrophobic carbodiimides such as DCCD, by partitioning in the lipid phase of the membrane can reach a local concentration sufficiently high to react covalently with a membrane protein functional group.

The inhibitory effect of dicyclohexylcarbodiimide (DCCD)* on the membrane ATPase of mitochondria was reported some years ago by Beechey et al. (1). Subsequently it was found that DCCD is a powerful inhibitor of the ATPase associated with the plasma membrane of Streptococcus fecalis (2). Active cation transport in this bacterium is also inhibited by this carbodiimide, a finding which along with other evidence (3,4,5,6) suggests that the membrane ATPase is involved in the energy transduction process. Consequently elucidation of the molecular basis of these inhibitory effects of DCCD could provide new insights concerning the mechanism of active transport. In earlier investigations we concluded that DCCD inhibited the bacterial membrane ATPase quite indirectly (2) since release of the inhibited

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*Abbreviations used: DCCD, dicyclohexylcarbodiimide

enzyme from its attachment to the membrane (7) resulted in a complete restoration of its catalytic activity. Moreover, DCCD had no effect on the soluble form of the enzyme while reattachment of the enzyme to depleted membranes restored its sensitivity to the inhibitor (2,7). These studies indicated that DCCD reacts with a membrane component, as yet unidentified, with which the enzyme is associated rather than with the enzyme itself. To account for the indirect inhibition of the membrane associated ATPase it has been suggested that the reaction of DCCD at some site in the membrane initiates conformation changes which are transmitted to the attached enzyme (2).

Not only is the identity of the DCCD-sensitive site in the bacterial membrane unknown, but neither do we know whether its reaction with DCCD is covalent or noncovalent. Evidence in favor of a covalent type reaction between DCCD and the sensitive site is the observation that the degree of inhibition of the membrane ATPase is time dependent and irreversible (2). The possibility of a covalent reaction of DCCD with a membrane protein as the basis for its inhibitory action is suggested from the many studies using well-defind pure proteins in aqueous solution, which show that a variety of water soluble carbodiimides can react covalently with protein functional groups such as -COOH, tyr-OH, $-NH_2$, -SH and ser-OH (8.9,10,11). However, this possibility may be questioned because DCCD is an effective inhibitor of the membrane ATPase at very low levels, for example, less than 0.01 µmole DCCD per mg membrane protein over a period of 15 min at pH 7.5. By contrast at least 1000 times more of the water soluble carbodiimide compounds are required for reaction with pure protein in aqueous solution.

To further explore the basis for the inhibition of the bacterial membrane ATPase by DCCD we have tested the effect of a number of other carbodiimides. If a covalent reaction were involved, such as a reaction with a membrane protein functional group, other carbodiimide compounds as well as DCCD might be expected to be inhibitory. It might be expected also

that the effectiveness of a particular carbodiimide as an inhibitor of the membrane ATPase would depend on its solubility in lipid if the DCCD-sensitive site happened to be situated within a non-polar region of the membrane. With these considerations in mind we selected some carbodiimides for testing which differed widely in their structure and water solubility.

RESULTS AND DISCUSSION

Table I shows the inhibitory effect on the Streptococcal membrane ATPase of 4 different carbodiimides tested over a 1000-fold range of concentrations. It can be seen that all the carbodiimides tested inhibited the membrane ATPase. It is apparent also that their relative effectiveness

TABLE I. INHIBITORY EFFECT OF VARIOUS CARBODIIMIDES ON BACTERIAL MEMBRANE ATPase

	% Inhibition			
	Concentration of Carbodiimide			
	$2x10^{-6}$ M	$2x10^{-5}$ M	$2x10^{-4}$ M	$2x10^{-3}$ M
DCCD	33	81*	89*	--
Di-p-tolycarbodiimide	19	37	73	--
diisopropylcarbodiimide	--	22	44	80
1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate	--	--	18	52

*This is the maximum inhibition that can be obtained since some of the membrane ATPase is resistant to inhibition by DCCD (2).

Membrane preparations were obtained by osmotic lysis of Strep. fecalis protoplasts (12,3). A sample of 0.25 mg dry weight of membranes were pre-incubated with the level of carbodiimide indicated for 15 min. at 25°C in 0.5 ml 0.1 M Tris Cl, pH 7.5, 2 mM $MgCl_2$. To assay for ATPase 0.5 ml of a solution of 200 mM Tris Cl, pH 7.5, 10 mM ATP and 8 mM $MgCl_2$ was then added and the mixture was incubated for an additional 10 min. at 38°C. The P_i liberated was determined as described previously (2).

differed by many orders of magnitude, with DCCD > di-p-tolycarbodiimide > diisopropylcarbodiimide > 1-cyclohexyl-3-(2 morpholinoethyl)-carbodiimide metho-p-toluene sulfonate. The potencies of these carbodiimides as inhibitors seem to be related to their non-polar character as judged by their structure and solubility in H_2O. Thus DCCD and di-p-tolycarbodiimide, both strong inhibitors, have large hydrocarbon substituents and are only slightly soluble in water. On the other hand, diisopropylcarbodiimide, with two short hydrocarbon chains, is readily soluble in both water and ethyl alcohol and is about 100 times less potent than DCCD. Finally, the least effective compound is a cationic carbodiimide, very soluble in H_2O and insoluble in ethyl alcohol. Presumably it would have a very low solubility in the lipid phase of a membrane, and in fact it is nearly 1000 times less potent as an inhibitor than DCCD. Here it should be mentioned that the widely different inhibitory potencies of those carbodiimides cannot be accounted for by differences in their stability in water since the experiments were performed at pH 7.5, a pH at which such compounds do not react readily with water (13). Furthermore, all of these carbodiimides appear to inhibit the membrane ATPase by the same mechanism in that they inhibited the membrane-bound but not the soluble form of the enzyme.

Since the experiments presented in Table I show that a variety of carbodiimides, in addition to DCCD, acted as inhibitors of the bacterial membrane ATPase, we conclude that the inhibition is the result of a covalent reaction of the carbodiimide group itself with the sensitive site on the membrane. The high sensitivity of the membrane ATPase system to low levels of the water-insoluble carbodiimides, when compared to the weak effect of the water-soluble reagents, suggests that the sensitive site is situated within the lipid phase of the membrane. It seems likely that by dissolving in the lipid phase of the membrane the hydrophobic carbodiimide compounds achieve an effective concentration sufficiently high to accomplish a covalent reaction with a protein functional group. Which of the possible functional

groups in a membrane protein might be attacked by the carbodiimides is an open question. The fact that DCCD is very effective at pH 7.5 would appear to exclude a carboxyl group since it is well known that carbodiimides activate only the non-ionized species. However, ionization of a carboxyl group would be suppressed when located in a hydrophobic region of the membrane and could therefore become susceptible to attack despite the high pH of the surrounding aqueous environment.

The membrane ATPase from both Streptococcus fecalis and in mitochondria have been isolated in a highly purified form and they are remarkably similar in their molecular properties such as amino acid composition, size and shape (14,15,16). In addition, both of these ATPases are inhibited by DCCD only when they are attached to their respective membranes. As far as we know there are no studies to show if the mitochondrial ATPase is inhibited by carbodiimide compounds, other than DCCD. However, by using C^{14}-DCCD Beechey (17) has provided evidence that this carbodiimide can react covalently with mitochondrial proteolipid. Our finding that carbodiimide compounds in general inhibit the bacterial membrane ATPase, taken together with the far greater potency of the more hydrophobic compounds is consistent with a covalent attack by these compounds on a proteolipid.

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