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INTERACTION OF THE DIBUTYLCHLOROMETHYLTIN CHLORIDE BINDING SITE WITH THE CARBODIIMIDE BINDING SITE IN MITOCHONDRIA.

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Summary. An examination of the effect of dibutylchloromethyltin/chloride on the carbodiimide binding proteolipid of mitrochondrial ATPase has revealed that in the presence of the alkyltin, (1) binding of dicyclohexycarbodiimide is decreased (2) the electron spin resonance spectrum of a nitroxide analogue of dicyclohexylcarbodiimide exhibits line broadening characteristic of either an increase of polarity or a decrease in viscosity of the carbodiimide binding site (3) the rate of reduction of the nitroxide probe by ascorbate is increased threefold. These phenomena suggest a possible mode of action for the inhibition of ATP synthesis by alkyltins.

DBCT[5] is a potent inhibitor of oxidative phosphorylation [1]. Previous studies [1,2] have shown that the site of action of DBCT is located in the oligomycin sensitive ATPase, and that the mode of action is similar to that of oligomycin and DCCD[5]. Both DBCT and DCCD react covalently with components of the oligomycin sensitive ATPase [2,3], and this provides the opportunity to study the mechanism of action of the inhibitors and the interaction (if any) between the reaction sites involved. Although the components binding DCCD and DBCT are different [2], our binding studies with [^{3}H] DBCT and [^{14}C] DCCD indicated that DBCT interfered with the binding of DCCD. This inter-

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5. Abbreviations: DBCT, dibutylchloromethytin chloride; DCCD, dicyclohexylcarbodiimide; NCCD, N-(2,2,6,6, - tetramethyl-piperidyl-1-oxyl)-N'-(cyclohexyl) carbodiimide.

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action has been studied by use of a spin probe analogue of DCCD, (NCCD)[5], which has been used to probe the viscosity and polarity of the carbodiimide binding site [4,5].

METHODS

Beef heart mitochondria were prepared by the method of Löw and Vallin [6] and suspended in 0.25 M sucrose, 10 mM Tris-chloride pH 7.6. Reaction with NCCD was as described by Azzi *et al.* [4]. NCCD-treated mitochondria were prepared by incubation of beef heart mitochondria at 0°C for 24 hours with 1 nmol NCCD/mg protein in 0.25 M sucrose, 50 mM Tris-chloride pH 7.5, 1mM EDTA, 1 mM potassium ferricyanide (incubation buffer). Unbound NCCD was removed by dialysis for 18 hours against 200 vols of incubation buffer containing 5 g Amberlite XAD-2 resin. This step was followed by 3 washes by resuspension in incubation and centrifugation at 40,000 g for 15 min.

Binding of DBCT to NCCD treated mitochondria was carried out by addition of 5-10 nmol DBCT/mg NCCD treated mitochondria in incubation buffer at 0°C for 18 hours. These preparations were used directly for ESR spectral determinations, and were not washed to remove unreacted DBCT.

ESR spectra were measured at ambient temperature (22°C) using a Varian E3 spectrometer. Reduction of the nitroxide by 1 mM ascorbate was performed in incubation buffer containing no ferricyanide. Methods for binding of [^{14}C]DCCD and [^{3}H]DBCT were as previously described (2).

RESULTS AND DISCUSSION

Binding studies with [^{14}C]DCCD and [^{3}H]DBCT showed that preincubation with DBCT reduced the binding of [^{14}C]DCCD (fig. 1). Incubation with 30 nmol DBCT/mg protein, at which concentration the DBCT binding site is fully titrated [2], reduces the binding of DCCD by 50%. This effect is unlikely to be due to competition for the same site or steric hindrance between the two inhibitors, as preincubation with an inhibitory concentration (1 nmol/mg protein) of DCCD had no effect on DBCT binding. Moreover, the component which binds DBCT is extractable by a wide range of organic solvents, unlike the carbodiimide binding proteolipid demonstrating the non identity of the binding sites. The decrease in DCCD binding was not due to a DBCT mediated release of bound DCCD as no radiolabel was released from [^{14}C]DCCD treated mitochondria on subsequent addition of DBCT.

Of interest in relation to the reduced binding are a similar reduction of [^{14}C]DCCD binding to the oligomycin sensitive ATPase in the presence of oxidative phosphorylation [7] and studies of the binding of [^{14}C]DCCD to yeast mitochondria [8], which show that other components of the ATPase, in

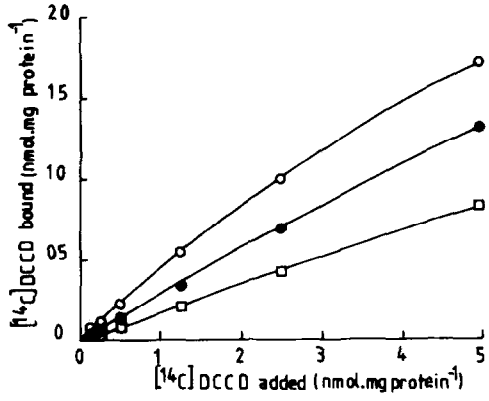

Figure 1 Binding of [^{14}C] DCCD to beef heart mitochondria. Beef heart mitochondria (4 mg) were incubated in 0.5 ml of 0.25 M sucrose, 10 mM Tris-Cl, 1 mM EDTA pH 7.6 in the presence of the indicated amounts of DBCT at 0°C for 8 hours. Varying amounts of [^{14}C] DCCD were added, and the suspension incubated for a further 18 hours at 0°C. The suspension was diluted to 5 ml with 0.25 M sucrose, 10 mM Tris-Cl, 1 mM EDTA pH 7.6 and centrifuged at 10,000 g for 15 min. The pellets were washed three times by resuspension in 5 ml 0.25 M sucrose, 10 mM Tris-Cl, 1 mM EDTA and centrifugation at 10,000 g for 10 min. The washed pellets were dissolved in 0.5 ml 10% SDS and counted in 5 ml of a toluene/triton X100/butyl PBD scintillation cocktail.

○——○ No DBCT

●——● 9.2 nmol DBCT/mg protein.

□——□ 27.6 nmol DBCT/mg protein.

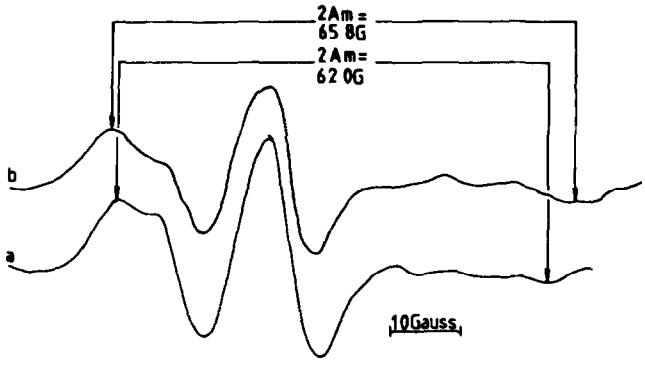

Figure 2 ESR spectrum of NCCD bound to beef heart mitochondria. Instrumental conditions were: microwave power 20 mW; modulation amplitude, 2.5 G; filter time constant 3S for a scan speed of 8 min.

(a) no further additions

(b) in the presence of DBCT at 10 nmol/mg protein.

addition to the carbodiimide binding protein, may play a role in the covalent binding of DCCD.

The availability of the spin probe NCCD, which is an analogue of DCCD and retains the inhibitory potency of the parent compound [4] enables investigation of the microenvironment of the carbodiimide binding site. The ESR spectrum of NCCD in aqueous media consists of three narrow lines of approximately equal amplitude [4], characteristic of a freely tumbling nitroxide. The spectrum of NCCD bound to beef heart mitochondria (fig. 2a) shows the line broadening typical of a strongly immobilised nitroxide as previously found for submitochondrial particles[4]. No change in the ESR spectrum of NCCD treated mitochondria was observed on addition of DBCT. However, after incubation at $0^{o}C$ for 18 hours, the distance between the outer lines (2 Am) increased from 62.0 G to 65.8 G (fig. 2b). No change was observed in NCCD treated mitochondria incubated in the absence of DBCT. The increase in line broadening on incubation with DBCT may be due to either further immobilisation of the bound NCCD, or to an increase in the polarity of the environment of the probe. The spectral parameters necessary to allow differentiation between these two possibilities cannot be derived for such a highly immobilised nitroxide [9].

The reduction of nitroxides by ascorbate may be usefully employed to indicate the orientation of a probe in the membrane [10, 11]. The reduction of NCCD bound to mitochondria is shown in Fig. 3, and was lower than that reported in submitochondrial particles[5], presumably due to oxidation of the abscorbate in the outer membrane. The rate of reduction of the nitroxide is three times faster in the presence of DBCT indicating an increased availability of the carbodiimide binding site to ascorbate. It is possible that the spectral changes observed in the presence of DBCT are due to an increase in polarity of the carbodiimide binding site, as found for removal of F_1 and OSCP from NCCD treated submitochondrial particles [5]. A decrease

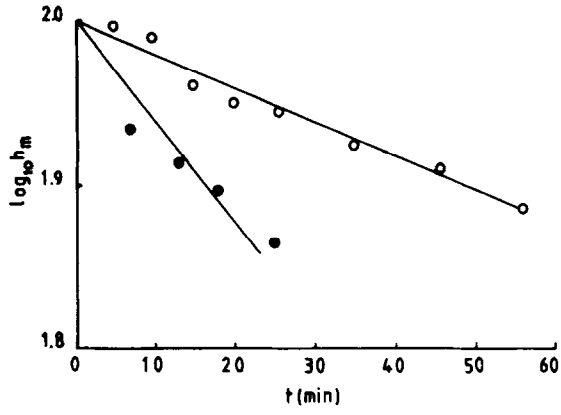

Figure 3 Reduction of NCCD by ascorbate. The spectra of NCCD bound to beef heart mitochondria were measured in the absence O——O and the presence ●——● of 10 nmol DBCT/mg protein. Ascorbate was added to a concentration of 1 mM and the lineheight of the central line of the spectrum measured at the times indicated.

in fluidity of the carbodiimide binding site would decrease the rate of reduction [11].

The data are consistent with a conformational change in the membrane sector of the proton translocating ATPase on addition of DBCT such that the carbodiimide binding site moves to a more polar environment. In view of the current hypothesis that the glutamic acid residue binding DCCD is an essential amino acid participating in the translocation of protons through the membrane sector of the ATPase [12,13], the mode of action of DBCT may be displacement of this glutamic acid to either an unfavourable environment for proton translocation, or away from other amino acid side chains involved in the reaction.

The decreased binding of DCCD in the presence of bound DBCT is accountable in terms of a reduced accessibility of the carbodiimide binding site. The carbodiimide binding proteolipid is normally situated in a region of the membrane accessible to nonpolar carbodiimides but not to polar, water soluble carbodiimides [14], and it is therefore probable that movement of the glutamic acid residue into a more polar environment would render it

less accessible to the nonpolar DCCD. Alternatively, the observed change to a more polar environment may increase the possibility of nucleophilic attack of the unstable o-acyl urea and loss of the inhibitor as dicyclohexyl-urea as suggested for decreased binding of DCCD on aerobiosis of S. cerevisise [9].

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