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CHARACTERIZATION OF DICYCLOHEXYLCARBODIIMIDE BINDING SITES IN BEEF-HEART MITOCHONDRIA

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SUMMARY: Binding studies with [^{14}C]-dicyclohexylcarbodiimide showed the presence of binding sites in the beef-heart mitochondrial membrane at a concentration of 1.8 nmol/mg protein (1.4 sites per cytochrome $a+a_3$). Saturation of these sites correlated with the inhibition of the ATPase activity. The maximum binding capacity could be related with the amount of F_1-ATPase in mitochondria from various tissues.

N, N´-dicyclohexylcarbodiimide (DCCD) is known as a specific inhibitor of both the hydrolytic and synthetic activities of the ATPase complex of mitochondria, bacteria and chloroplast (1-4). However, the total inhibition of the ATPase activity is achieved at DCCD concentrations significantly lower than those required for saturation of the specific binding sites on the DCCD-binding protein (4, 5). It was suggested that the DCCD-binding protein exists as an oligomer, most probably as a hexamer and that occupation of only a single monomer is sufficient for the inhibition of the enzyme activity (5, 6). In order to obtain further data concerning this problem, the DCCD binding sites on the beef--heart and brown-adipose tissue mitochondria were studied and the binding parameters were compared with the inhibition of the ATPase activity.

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MATERIALS AND METHODS

Preparation of mitochondria and analytical procedures: Beef-heart mitochondria were prepared according to Smith(7). Brown-adipose tissue mitochondria were isolated from brown--adipose tissue of Syrian hamsters by the method of Hittelman et al. (8). Prior to all measurements, mitochondria were disrupted by freezing-thawing 3 times followed by a 15 min centrifugation at 30 000 g. Sedimented mitochondrial membranes were suspended in 0.25 M sucrose, 10 mM TRIS-HCl, 1 mM EDTA, pH 7.4. The content of cytochromes was determined according to Williams (9). ATPase activity was measured as inorganic phosphate released (10) during a 3 min incubation at 30°C in a medium containing 50 mM KCl, 10 mM TRIS-HCl, 3 mM $MgCl_2$, 5 mM ATP, pH 7.4, as described before (11). Proteins were determined by the method of Lowry (12).

[^{14}C]-DCCD binding study: Beef heart or brown adipose tissue mitochondria suspended in 0.25 M sucrose, 10 mM TRIS, 1 mM EDTA, pH 7.4 (about 0.25 mg protein/mg) were incubated with various [^{14}C]-DCCD concentrations for 16 h at 4°C. [^{14}C]-DCCD was added as an ethanolic solution up to 16 µl per ml of the mitochondrial suspension. When determining the bound [^{14}C]-DCCD label, the incubation mixture was filtered through a single Whatman GF/C filter and the mitochondrial membranes left on the filter were washed at a very low flow-rate (50-60 ml/min) with 15 ml of the following solutions:(a) 0.25 M sucrose, 10 mM TRIS, 1 mM EDTA, pH 7.4, (b) 5% TCA, (c) 5% TCA + 5 mM non-labelled DCCD, (d) 10% water in acetone. As much as 92-95% of the mitochondrial protein was recovered on the filters. The filters were dried overnight at laboratory temperature and the radioactivity was determined in a liquid scintillation system using TOLUENE : TRITON X-100 (2 : 1). The distribution of the [^{14}C]-DCCD obtained on the basis of the equilibrium binding studies was analysed according to Scatchard (13) using a Hewlett-Packard 21 programable computer.

RESULTS AND DISCUSSION

Beef heart mitochondria were incubated with increasing concentrations of [^{14}C]-DCCD and the [^{14}C]-label bound to the mitochondrial membranes washed with sucrose-TRIS-EDTA, TCA or TCA + non-radioactive DCCD was estimated as described in Methods. The binding curve presented in Fig. 1 indicates that the saturation of the DCCD binding sites was not obtained within the [^{14}C]-DCCD concentration range used.

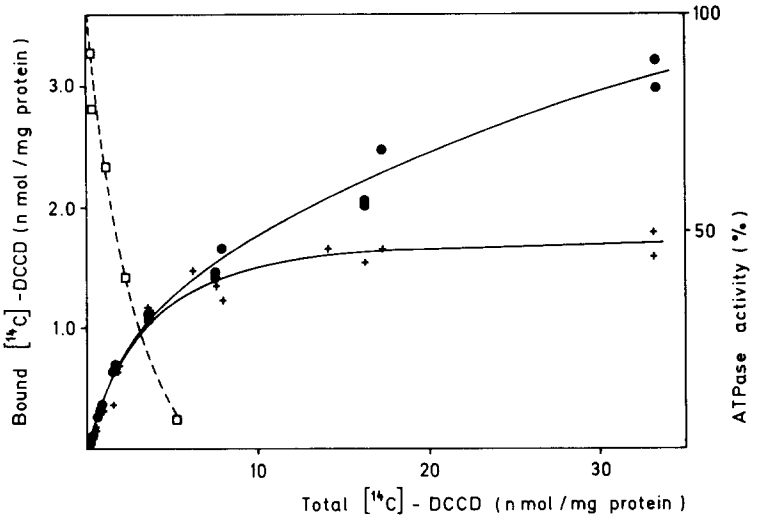

Figure 1 [^{14}C]-DCCD binding to beef-heart mitochondria as a function of increasing concentrations of [^{14}C]-DCCD: Frozen-thawed mitochondria were incubated with [^{14}C]-DCCD and the bound [^{14}C]--label was determined after washing with a 0.25 M sucrose, 10 mM TRIS-HCl, 1 mM EDTA, pH 7.4, with a 5% TCA or with a 5% TCA + 5 mM non-radioactive DCCD (●-●). There was no difference in the binding data obtained with these media and the results were therefore plotted in one binding curve. A 5% water in aceton (x-x) was used for washing. Inhibition of ATPase activity by DCCD (□---□) was measured simultaneously with the determination of the bound [^{14}C]-label and the inhibition was expressed as percent of activity without DCCD.

When acetone-water was used for washing the mitochondrial membranes, the shape of the binding curve was modified. The DCCD binding at low concentrations was not affected whereas the binding at higher DCCD concentrations was considerably suppressed,so that saturation of binding sites was obtained (Fig. 1).

A solution of 10% water in acetone is known to extract majority of the mitochondrial phospholipids (14). It was shown that [^{14}C]-DCCD binds non-specifically to mitochondrial

phospholipids (15), and, hence, it could be concluded that the extraction of mitochondrial phospholipids decreased the total bound [^{14}C]-label by the amount of [^{14}C]-DCCD bound non-specifically to phospholipids.

Parallel measurements of the ATPase activity revealed that the half-maximum inhibition of the enzyme activity was reached at a 0.3 μM concentration, i.e. at 1.5 n-mole DCCD/mg protein. In these conditions 0.6 nmole of [^{14}C]-DCCD was bound per mg of mitochondrial protein. A complete inhibition of the ATPase activity was obtained when 1.2 nmole of the [^{14}C]--DCCD was bound per mg protein. Comparison of the enzyme activity and [^{14}C]-DCCD binding showed that DCCD is further bound even when maximum inhibition of ATPase activity is reached (Fig. 1).

For evaluation of maximum number of binding sites (B_{MAX}) and determination of apparent dissociation constant (K_D), the data of [^{14}C]-DCCD binding were analysed according to Scatchard (13) . It follows from Fig. 2 that the Scatchard plot derived from samples washed with sucrose-TRIS-EDTA, TCA or TCA + unlabelled DCCD was curvilinear with upward concavity. No intercept with abscissa was reached within the concentration range studied. This indicates the presence of multiple binding sites with higher and lower affinities towards the ligand (16). On the contrary, the Scatchard plot derived from samples washed with the acetone-water solution was linear with negative slope. The intercept of this plot with the ordinate gives a value of B_{MAX} 1.8 nmol DCCD/mg protein (1.4 mol DCCD/mol cytochrome a + a_3). Apparent dissociation constant K_D calculated from the slope of this plot and the B_{MAX} value was 0.45×10^{-6}M. Comparison of both Scatchard plots indicates

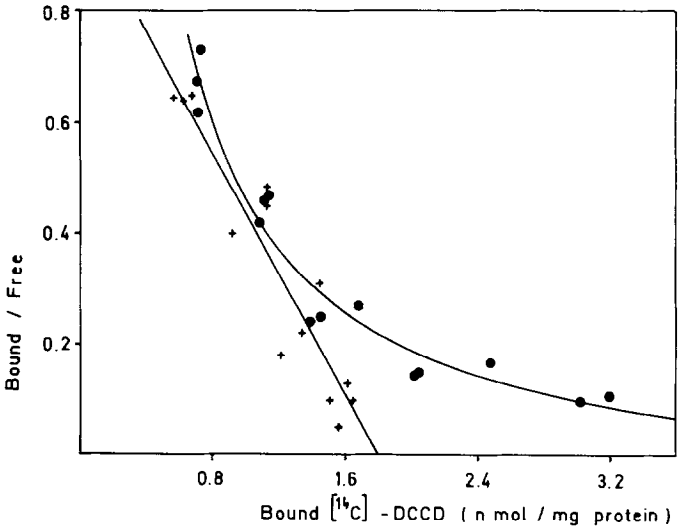

Figure 2 Scatchard plot of [^{14}C]-DCCD binding to beef--heart mitochondria: The binding data presented in Fig. 1 were analysed according to Scatchard (13). Mitochondria were washed with a 0.25 M sucrose,10 mM TRIS-HCl pH 7.4, 1 mM EDTA, with a 5% TCA, with a 5% TCA + 5 mM non-radioactive DCCD (●-●) or with a 5% water in aceton (x-x).

that after the extraction of mitochondrial phospholipids,the non-saturable portion of [^{14}C]-DCCD binding was removed and that only one type of [^{14}C]-DCCD binding sites appeared under these experimental conditions. These results also show that non-specific [^{14}C]-DCCD binding sites on phospholipids described by Dianoux et al. (15) exhibit a lower affinity than those on proteins. The apparent K_D of specific DCCD binding sites 0.45 μM corresponded well to the DCCD concentration required for a 50% inhibition of ATPase (0.3 μM). The maximum number of specific DCCD binding sites B_{MAX} was 1.5 times higher than the amount of DCCD bound at 96% inhibition of ATPase activity (see Fig. 1) which also shows that the ATPase inhibition is correlated with the saturation of specific binding sites. Furthermore, when the B_{MAX} values were determined

in mitochondrial membranes differing significantly in the content of F_1-ATPase, clear correlation between the capacity of high-affinity binding sites and the amount of F_1-ATPase occured. In comparison with beef-heart mitochondria, the specific DCCD binding sites in the brown-adipose tissue mitochondria (0.2 nmole/mg protein) were reduced proportionally with the reduced content of F_1-ATPase, i.e. 10 times (17).

Therefore, it can be concluded that both binding parameters of specific DCCD-binding sites, B_{MAX} and apparent K_D, correlate with the sensitivity of ATPase towards this inhibitor or content of ATPase in the mitochondrial membrane. Thus, it appears that the DCCD binds in the beef-heart mitochondria to a heterogenous group of binding sites, where the high and low-affinity types may be distinguished. The data suggest that the saturation of high-affinity sites is primarily involved in the ATPase complex inhibition.

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