Reactivity of Mitochondrial F_1-ATPase to Dicyclohexylcarbodiimide. Inactivation and Binding Studies†

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ABSTRACT: There are two sites of action of DCCD on the mitochondrial ATPase complex, the membrane sector and the F_1-ATPase. Incubation of F_1-ATPase isolated from beef heart mitochondria with dicyclohexylcarbodiimide (DCCD) resulted in inactivation of the enzyme. The DCCD inactivation of F_1-ATPase was both time and concentration dependent. Kinetic data indicated that 1 mol of DCCD binds to 1 mol of active site. Inactivation of F_1-ATPase by DCCD was pH dependent, being more marked at acid pH. Half-maximal effect was around pH 7.5, no inactivation occurring above pH 8.5. The half-time of inactivation was decreased by 20% by 10 mM ATP and ADP and increased by 50% by 10 mM $MgCl_2$. Complete inactivation of F_1-ATPase required the binding of 2 mol of [^{14}C]DCCD per mol of F_1-ATPase. The binding sites of [^{14}C]DCCD were located on the β subunit of F_1-ATPase. Two other carboxyl reagents which inhibit the ATPase activity, *N*-cyclohexyl-*N'*-β-(4-methylmorpholine)-ethylcarbodiimide and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, did not interfere with DCCD binding. Glycine ethyl ester, a nucleophile compound which reacts selectively with carbodiimide-activated carboxyl groups, removed half of the bound [^{14}C]DCCD. The DCCD-modified F_1-ATPase gave a fluorescent complex with aurovertin similar to the unmodified F_1-ATPase. However, in contrast to the F_1-ATPase–aurovertin complex, the fluorescent intensity of the DCCD-modified F_1-ATPase–aurovertin complex was no longer quenched by ATP and $MgCl_2$ and no longer enhanced by ADP.

The Mg^{2+}-activated ATPase complexes found in membranes of mitochondria, chloroplasts, and bacteria are made of two sectors (for reviews, see Senior, 1973; Pedersen, 1975; Mitchell, 1976; Nelson, 1976; Panet & Sanadi, 1976; Haddock & Jones, 1977). The first sector designated as F_1 (mitochondria), CF_1 (chloroplasts), or BF_1 (bacteria) is easily detachable from the membrane, whereas the second one is an integral part of the membrane. ATPase inhibitors can be divided into two classes, depending on their action on either of the two sectors. Dicyclohexylcarbodiimide (DCCD)[1] is a well-known inhibitor of the membrane-bound ATPases (Beechey, 1974). The binding of DCCD to the membrane sector of various ATPases has been extensively studied and the DCCD-binding protein has been characterized as a highly hydrophobic peptide of molecular weight 8000–13000 (Cattell et al., 1971; Stekhoven et al., 1972; Fillingame, 1975; Altendorf & Zitzmann, 1975; Nelson et al., 1977; Sebald et al., 1977; Wachter et al., 1977). On the other hand, there are only brief mentions of the inhibitory effect of DCCD on the F_1-ATPase (Penefsky, 1967; Beechey et al., 1975). This paper describes the inhibitory effects of DCCD on the hydrolytic activity of beef heart F_1-ATPase and correlative binding studies with [^{14}C]DCCD. It is concluded that DCCD binds to the β subunit of F_1-ATPase, full inactivation being achieved when 2 mol of DCCD is bound per mol of F_1, and that DCCD binding to F_1-ATPase counteracts the interaction of ATP with the nucleotide binding site(s) of F_1-ATPase.

Experimental Section

Materials

Aurovertin D was purified from cultures of *Calcarisporium arbuscula* (NRRL 3705) (Osselton et al., 1974) and stored at −20 °C as an ethanolic solution protected from light. The molar extinction coefficient of our aurovertin D preparation was determined to be 35100 at 368 nm. Aurovertin fluorescence was measured at 25 °C with a Perkin-Elmer MPF2A fluorimeter. The medium was made of 0.25 M sucrose, 10 mM Tris-HCl, 0.5 mM EDTA, pH 7.4. The excitation wavelength was set at 365 nm and the emission wavelength at 470 nm.

[^{14}C]DCCD (54.5 Ci/mol) was obtained from the Commissariat à l'Energie Atomique (CEA, Saclay). It was more than 97% radiochemically pure as judged by thin-layer chromatography (Cattell et al., 1971) followed by autoradiography. It showed concentration vs. inhibition curves identical with that of unlabeled DCCD. Unlabeled DCCD was freed of any dicyclohexylurea according to the procedure described by Fieser & Fieser (1967). DCCD was used as a methanolic solution and appropriate controls were run with methanol alone (final concentration: 2%).

ATP, ADP, and phosphoenolpyruvate were purchased from Boehringer. All other products were of reagent grade quality.

Methods

Preparation of Beef Heart Mitochondrial ATPase. Beef heart mitochondria were isolated by the method of Smith (1967). Beef heart mitochondrial F_1-ATPase was prepared and stored as an ammonium sulfate suspension as described by Knowles & Penefsky (1972). Its specific activity varied between 80 and 100 μmol min^{-1} (mg of protein)$^{-1}$ under the described assay conditions. Molar concentration of purified F_1-ATPase was based on a molecular weight of 360000 (Lambeth et al., 1971). The enzyme used for inactivation assays was freed of ammonium sulfate by the elution–centrifugation method described by Penefsky (1977). The ammonium sulfate precipitate of F_1 was dissolved in the required buffer and 0.1 mL of the enzyme solution was applied to the top of a small column of Sephadex G-50 (fine) which

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[1] Abbreviations used: DCCD, *N*,*N'*-dicyclohexylcarbodiimide; CMCD, *N*-cyclohexyl-*N'*-β-(4-methylmorpholine)ethylcarbodiimide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; NBD, 4-chloro-7-nitrobenzofurazan; Mops, 3-(*N*-morpholino)propanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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was made in a 1-mL plastic tuberculin syringe and inserted in a centrifuge tube (Penefsky, 1977) The eluate excluded after centrifugation was used for ATPase assays.

ATPase Activity Determination. Most of the measurements of ATPase activity were carried out at 30 °C by determination of the inorganic phosphate released, as described previously (Pougeois et al., 1978). The reaction medium (final volume: 0.5 mL) contained 40 mM Tris-HCl, 10 mM ATP, 5 mM $MgCl_2$, 20 μg of pyruvate kinase, and 2 mM phosphoenolpyruvate (final pH 8.0). The reaction was started by addition of an aliquot fraction of the F_1-ATPase solution (1–3 μg) and stopped after 5 min by addition of 0.2 mL of trichloroacetic acid, 50% (w/v). The released inorganic phosphate was determined by the Fiske & Subbarow method (1925).

When the ATPase activity had to be determined at low ATP concentrations (for example, calculation of K_m^{ATP}), a spectrophotometric method was used. The reaction mixture in 2 mL contained 50 mM Tris-HCl, 240 mM sucrose, 0.2 mM NADH, 1 mM free magnesium (as $MgCl_2$), 2 mM phosphoenolpyruvate, 50 μg of pyruvate kinase, 25 μg of lactate dehydrogenase, 10 mM KCl, 10 mM $NaHCO_3$ (final pH 8.0), and different concentrations of Mg–ATP. The reaction was carried out and monitored at 30 °C by disappearance of NADH at 340 nm.

[^{14}C]DCCD Binding Experiments. After incubation of F_1-ATPase with [^{14}C]DCCD, the [^{14}C]DCCD-bound ATPase was freed from unreacted [^{14}C]DCCD by the elution–centrifugation method described by Penefsky (1977) (see above). The values for radioactivity in the eluate were those of the bound [^{14}C]DCCD. They were corrected for background by omission of the enzyme in the reaction mixture.

Acrylamide Gel Electrophoresis. Electrophoresis in 10.3% polyacrylamide gels containing 0.1% sodium dodecyl sulfate was carried out as described by Weber & Osborn (1969). After migration the gels were placed in 50% trichloroacetic acid for 3–4 h with one change for protein fixing, stained by 0.25% Coomassie Blue R-250, 20% trichloroacetic acid for 1 h, and then destained in 7% acetic acid. The densitometric traces were recorded with a Joyce-Loebl Scan 400. To determine the distribution of radioactivity in the gels, the gels previously fixed, stained, and destained were frozen in solid CO_2 and sliced into 1-mm slices with a Joyce-Loebl gel slicer. Slices were digested by overnight incubation in 1 mL of 15% H_2O_2 at 55–60 °C and counted in 10 mL of a scintillation fluid (Patterson & Greene, 1965).

Protein Assay. The protein concentration of ATPase preparations was determined with the Folin-Ciocalteu reagent using bovine serum albumin as a standard in the same buffer as the ATPase (Zak & Cohen, 1961).

Results

Kinetics of Inactivation of F_1-ATPase by DCCD. Incubation of F_1-ATPase with DCCD resulted in a progressive decrease in enzyme activity. DCCD inhibition was irreversible and ATPase activity could not be restored by gel filtration or dilution of F_1-ATPase.

Inactivation of F_1-ATPase by DCCD was both time and concentration dependent. For DCCD concentrations ranging between 0.02 and 1 mM, the time course of inactivation followed pseudo-first-order kinetics until 80–85% of activity was lost (see insert in Figure 1). Below 5×10^{-3} mM DCCD, virtually no inhibition was found. On the other hand, concentrations of DCCD higher than 0.2 mM brought about a similar rate of inactivation. The inhibition data were treated by the method of Levy et al. (1963) to calculate the number of DCCD molecules able to react per active site of F_1-ATPase

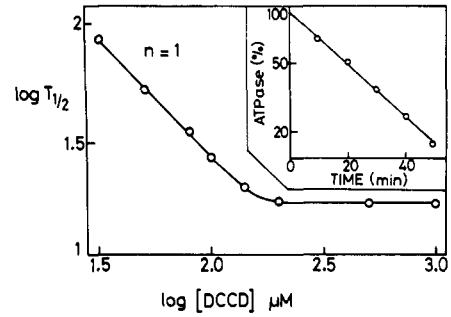

FIGURE 1: Number of reactive DCCD molecules per active site of F_1-ATPase. F_1-ATPase (0.7 mg/mL) was preincubated at 24 °C in a series of tubes containing 0.1 mL of a medium made of 50 mM Tris, 50 mM Mops, 2 mM EDTA, 4 mM ATP, final pH 7.0, and increasing concentrations of DCCD. Aliquot samples were assayed at various times for ATPase activity as described in Methods. The half-time of inactivation ($T_{1/2}$, min) was calculated for each DCCD concentration (see insert). The data were plotted as log ($T_{1/2}$) against the logarithm of DCCD concentration. The number n stands for the number of DCCD binding sites. The insert shows a typical semilogarithmic plot of the time course of inactivation by 140 μM DCCD.

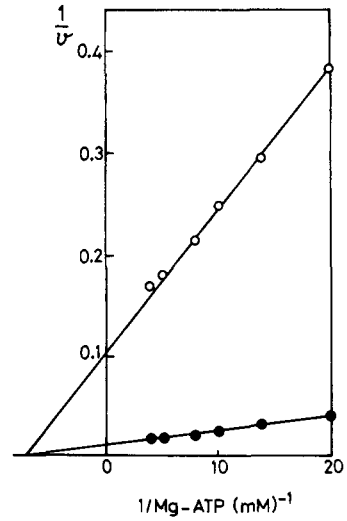

FIGURE 2: Kinetic analysis of the inhibitory effect of DCCD on F_1-ATPase. F_1-ATPase was preincubated for 40 min with 0.2 mM DCCD as detailed in the legend to Figure 1. A control incubation was run under identical conditions without DCCD. Then, each medium was subjected to the chromatography–centrifugation procedure described in Methods. The ATPase activities of the control enzyme (●) and the DCCD-modified enzyme (○) recovered in the eluates were measured spectrophotometrically at 30 °C (cf. Experimental Section). K_m^{ATP} was in both cases 0.14 mM. Velocities are given in μmol of inorganic phosphate released min^{-1} (mg of protein)$^{-1}$.

(Figure 1). The plot of log $T_{1/2}$ against log DCCD concentration gave a straight line with a slope close to 1 for concentrations of DCCD below 0.2 mM suggesting that the binding of 1 mol of inhibitor to 1 mol of active site eliminates the enzyme activity. Therefore ATPase inactivation by DCCD is probably due to a simple modification of an essential amino acid residue (cf. Levy et al., 1963). Above 0.2 mM DCCD, the slope of the curve (Figure 1) tended toward zero, indicating a saturation process most likely related to the low solubility of DCCD in the incubation medium.

Double-reciprocal plots of ATPase activity vs. Mg–ATP concentration indicated that both DCCD-treated F_1-ATPase and untreated F_1-ATPase have the same K_m (Figure 2), which is in accordance with an all-or-none inactivating effect of DCCD on a fraction of F_1-ATPase molecules.

Inactivation of F_1-ATPase by DCCD was pH dependent, being more marked at acid pH. Half-maximal effect was

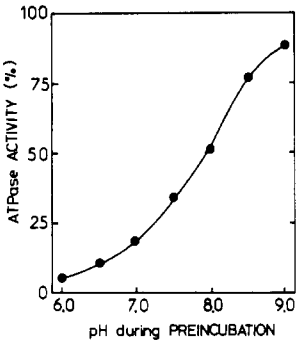

FIGURE 3: pH dependence of the DCCD inactivation of F_1-ATPase. F_1-ATPase (0.4 mg/mL) was preincubated for 60 min at 24 °C with 0.2 mM DCCD in 50 mM Mops, 50 mM Tris, 2 mM EDTA, and 4 mM ATP at the indicated pH. Final volume: 0.1 mL. Then aliquot samples were assayed for ATPase activity.

Table I: Effect of ATP, ADP, and Mg^{2+} on Inactivation of F_1-ATPase by DCCD[a]

additions	half-time of inactivation (min)
none	31
10 mM ATP	25
10 mM ADP	25
10 mM $MgCl_2$	45

[a] F_1-ATPase (0.7 mg/mL) was preincubated at 24 °C with 0.14 mM DCCD in 50 mM Tris, 50 mM Mops, 2 mM EDTA, final pH 7.0. Aliquot samples were assayed at intervals for ATPase activity (see the Experimental Section).

found around pH 7.5. There was virtually no inactivation above pH 8.5 (Figure 3). Both DCCD-treated F_1-ATPase and untreated F_1-ATPase have the same pH profile of activity with a similar broad peak in the region of pH 8.5–9. Thus, the decreased activity caused by reaction of DCCD is not due to a shift of optimum pH in the modified ATPase.

Protection against DCCD Inactivation. The effect of ATP, ADP, and Mg^{2+} on DCCD inactivation is shown in Table I. The half-time of inactivation was decreased by 10 mM ATP or ADP by about 20% and on the contrary increased by 10 mM $MgCl_2$ by about 50%.

Inactivation of F_1-ATPase by DCCD was found to be insensitive to the addition of the following nucleophilic compounds used at the final concentration of 2 mM: hydroxylamine, hydrazine, dithiothreitol, 2-merc ptoethanol. This is in contrast to the protective effect of these compounds against inactivation of F_1-ATPase by EEDQ, another carboxyl group reagent (Pougeois et al., 1978).

[^{14}C]DCCD Binding to F_1-ATPase. Identification of the Binding Subunit. A direct measurement of DCCD-binding sites in F_1-ATPase was performed with [^{14}C]DCCD. The linear relationship between the binding of [^{14}C]DCCD to F_1-ATPase and the loss of ATPase activity is illustrated in Figure 4. By extrapolating binding data, it could be calculated that complete inactivation of F_1-ATPase requires the binding of 2 mol of [^{14}C]DCCD.

To determine to which subunit of F_1-ATPase [^{14}C]DCCD binds covalently, the enzyme after inactivation by [^{14}C]DCCD was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The profile of radioactivity given in Figure 5 shows essentially one peak of radioactivity with an apparent molecular weight of 50 000, corresponding to the β subunit of F_1-ATPase.

Effect of CMCD and EEDQ on the Binding of [^{14}C]DCCD to F_1-ATPase. Subsequent experiments were carried out to check whether CMCD and EEDQ, which both are known to

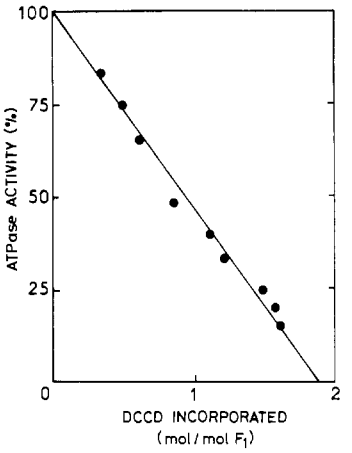

FIGURE 4: Correlation of inactivation of F_1-ATPase with incorporation of [^{14}C]DCCD. Inactivation of F_1-ATPase was performed with 0.2 mM DCCD as described in the legend to Figure 1. Aliquots were removed at intervals and subjected to the chromatography–centrifugation procedure (cf. Methods) before being assayed for ATPase activity and [^{14}C]DCCD incorporation. Control assays were carried out under the same conditions with [^{14}C]DCCD and without enzyme (radioactive blanks).

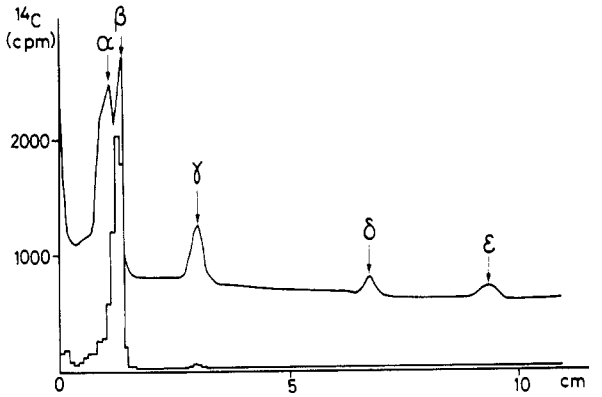

FIGURE 5: $NaDodSO_4$ gel electrophoresis of F_1-ATPase after labeling by [^{14}C]DCCD. F_1-ATPase (1 mg of protein) was treated for 2 h with 0.2 mM [^{14}C]DCCD as detailed in the legend to Figure 1 except that the pH was 7.5. The enzyme was inactivated to 85%. After precipitation by ammonium sulfate (50% final concentration), the pellet was solubilized in 40 mM Tris-HCl, 2 mM EDTA, 4 mM ATP (pH 7.5) and dialyzed for 1 h at 20 °C against the same buffer. A sample (70 μg) was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After staining and destaining, the gel was scanned and the distribution of radioactivity was determined. For details see the Experimental Section.

react with carboxyl group(s) in F_1-ATPase (Kozlov & Skulachev, 1977; Pougeois et al., 1978), interfere with the binding of [^{14}C]DCCD to F_1-ATPase. The enzyme was first left to react with CMCD or EEDQ until activation reached about 80%. Then [^{14}C]DCCD was added. After a given time of reaction with [^{14}C]DCCD, the reaction was terminated by the gel filtration–centrifugation procedure (cf. Methods), and the bound ^{14}C radioactivity was determined in the eluate. As shown in Table II, [^{14}C]DCCD was incorporated almost to the same extent in control F_1-ATPase, in CMCD-treated F_1-ATPase, and in EEDQ-treated F_1-ATPase, suggesting that DCCD binds to a different site than CMCD and EEDQ.

Effect of Glycine Ethyl Ester on the Binding of [^{14}C]DCCD to F_1-ATPase. Glycine ethyl ester has proved to be useful to detect a selective binding of carbodiimide to carboxyl groups. In fact, glycine ethyl ester reacts rather selectively with a carbodiimide-activated carboxyl group to give a stable carboxyl derivative (Khorana, 1953; Hoare & Koshland, 1967; Carraway & Koshland, 1972). The rate of inactivation of F_1-

Table II: [^{14}C] DCCD Fixation on CMCD- and EEDQ-Inactivated F_1-ATPase[a]

incubation time (min)	mol of [^{14}C] DCCD/ mol of F_1	mol of [^{14}C] DCCD/ mol of "CMCD-F_1"	mol of [^{14}C] DCCD/ mol of "EEDQ-F_1"
0	0	0	0
10	0.87	0.76	0.75
20	1.38	1.19	1.25
30	1.78	1.65	1.50

[a] A preparation of F_1-ATPase in 50 mM Mops, pH 6.5, and 2 mM EDTA (concentration: 0.5 mg of protein/mL) was divided into three fractions. Fraction 1 was the control. Fractions 2 and 3 were treated at 24 °C by 9 mM CMCD and 0.5 mM EEDQ, respectively, to obtain an inactivation of about 80%. The three fractions were treated identically by the chromatography–centrifugation technique described in the Experimental Section, using a chromatography column equilibrated with 50 mM Mops, 2 mM EDTA, and 4 mM ATP, pH 6.5. The eluates freed of unreacted CMCD or EEDQ were incubated with 0.2 mM [^{14}C] DCCD at 24 °C for the time indicated in the table and then aliquot fractions (0.1 mL) were subjected to another chromatography–centrifugation to recover the [^{14}C] DCCD-bound F_1 freed of the unreacted [^{14}C]-DCCD.

Table III: Inactivation and Incorporation of [^{14}C] DCCD in F_1-ATPase in the Presence of Glycine Ethyl Ester[a]

incubation time (min) with [^{14}C]-DCCD	without glycine ethyl ester: % remaining act.	without glycine ethyl ester: bound [^{14}C]-DCCD/ F_1-ATPase (mol/mol)	with 0.2 M glycine ethyl ester: % remaining act.	with 0.2 M glycine ethyl ester: bound [^{14}C]-DCCD/ F_1-ATPase (mol/mol)
10	60	0.93	57	0.53
20	42	1.28	39	0.63
30	28	1.73	26	0.76

[a] An aliquot fraction (0.4 mL) of F_1-ATPase (0.5 mg of protein/mL) in 50 mM Mops, 2 mM EDTA, 4 mM ATP, pH 6.5, was preincubated with 0.2 M glycine ethyl ester for 1 min at 24 °C. A control incubation was run without glycine ethyl ester. Then 0.2 mM [^{14}C] DCCD was added in both samples and left to react for the periods of time indicated in this table. After incubation, aliquot fractions (0.1 mL) were subjected to the chromatography–centrifugation procedure described in the Experimental Section to recover the [^{14}C] DCCD bound F_1-ATPase.

ATPase was not modified by addition of glycine ethyl ester up to a concentration of 250 mM (Table III). Above this concentration, F_1-ATPase activity decreases significantly. Incorporation of [^{14}C]DCCD was roughly halved by glycine ethyl ester at concentrations ranging between 1 and 250 mM (Figure 6). The 50% decrease in [^{14}C]DCCD binding induced by glycine ethyl ester was independent of the incubation period with the enzyme. Thus there are probably two distinct DCCD-reactive groups in the F_1-ATPase which differ in their respective reactivity toward glycine ethyl ester; one of them can be identified as a carboxyl group.

In proteins, DCCD may bind to amino acid residues other than carboxyl groups (Kurzer & Douraghi-Zadeh, 1967; Carraway & Koshland, 1972). No systematic attempt was carried out to determine the nature of the other DCCD-reactive group in F_1-ATPase. The binding of DCCD to a tyrosine residue of F_1-ATPase was eliminated because the bound DCCD was not released by a 2-h incubation at 24 °C with 0.5 M NH_2OH (pH 7.5), conditions which are known to decompose the derivatives formed by reaction of carbodiimide with the phenolic group of tyrosine (Carraway & Koshland, 1972).

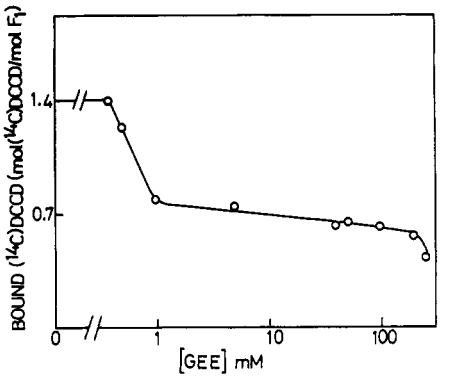

FIGURE 6: Effect of glycine ethyl ester on the binding of [^{14}C]DCCD by F_1-ATPase. F_1-ATPase was preincubated for 30 min with [^{14}C]DCCD (0.2 mM final concentration) as described in the legend to the Figure 1 (except that the pH was 6.5) and in the presence of various concentrations of glycine ethyl ester. At that time the enzyme was inactivated to an extent of 65%. The [^{14}C]DCCD incorporation was measured on aliquots of the enzyme which had been subjected to the chromatography–centrifugation procedure.

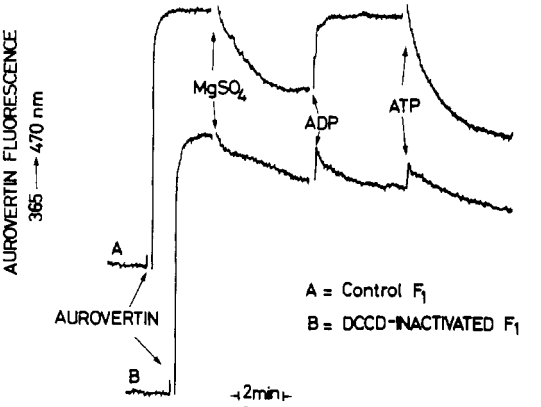

FIGURE 7: Fluorescence of the complex aurovertin–DCCD inactivated F_1-ATPase. F_1-ATPase (0.7 mg/mL) was preincubated for 45 min at 25 °C with 0.2 mM DCCD in 50 mM Mops, 2 mM EDTA, 4 mM ATP, pH 6.5. The enzyme was recovered free of DCCD by the chromatography–centrifugation procedure (cf. Methods). The fluorescence was carried out at 25 °C on 0.15 mg of active or DCCD-inactivated F_1-ATPase in 2 mL of 0.25 M sucrose, 10 mM Tris-HCl, 0.5 mM EDTA, pH 7.4. As indicated by arrows, the following compounds were added to the final concentration of 0.4 μM (aurovertin), 2.5 mM ($MgSO_4$), 0.1 mM (ADP), and 2 mM (ATP).

Aurovertin Binding to DCCD-Inactivated F_1-ATPase. When aurovertin binds to F_1-ATPase, its fluorescence is markedly increased (Lardy & Lin, 1969; Chang & Penefsky, 1973); the fluorescence of the aurovertin–F_1-ATPase complex is quenched by ATP (Chang & Penefsky, 1973). DCCD did not prevent the fluorescence increase caused by the binding of aurovertin to F_1-ATPase, indicating that the binding of DCCD to F_1-ATPase does not suppress the binding of aurovertin. However, the fluorescence of the complex made by aurovertin and the DCCD-inactivated F_1-ATPase was no longer quenched by $MgCl_2$ and ATP, or enhanced by ADP (Figure 7).

Discussion

There are two main sites of action of DCCD on the ATPase complex of heart mitochondria. The first one, identified by Cattell et al. (1971) and Stekhoven et al. (1972) is a hydrophobic protein (molecular weight 10000–13000) of the membrane sector of the ATPase complex. The data presented in this paper indicate that the second site of action of DCCD is the β subunit of the hydrophilic F_1 sector of the ATPase complex. However, the F_1 sector is about ten times less

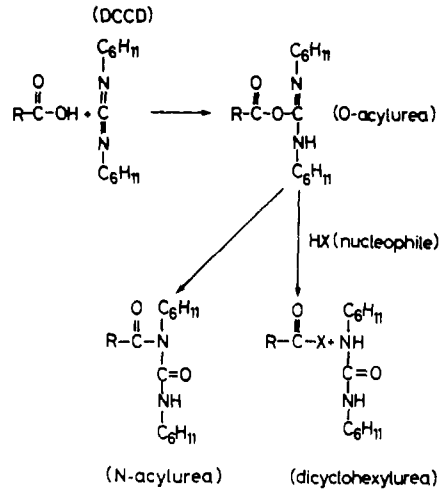

FIGURE 8: Reaction scheme of DCCD with carboxyl groups.

sensitive to DCCD than the membrane sector of the ATPase complex. The binding of DCCD to the β subunit of F_1-ATPase is accompanied by an inactivation of the enzyme. DCCD can therefore be added to a list of ligands including 4-chloro-7-nitrobenzofurazan and aurovertin whose specific binding to the β subunit of F_1-ATPase results in a loss of ATPase activity (Ferguson et al., 1975; Verschoor et al., 1977).

The fact that DCCD inhibits the effects of ATP, ADP, and Mg^{2+} on the fluorescence of the aurovertin-F_1-ATPase complex suggests either that DCCD binds to a site which is located close to those of ATP, ADP, and Mg^{2+} or that it prevents a change of conformation which is induced by these ligands and which is propagated to the aurovertin site. Two other carboxyl reagents, CMCD (Kozlov & Skulachev, 1977) and EEDQ (Pougeois et al., 1978), which inhibit F_1-ATPase are likely to bind to a carboxyl group different from that which reacts with DCCD since their binding does not interfere with the binding of DCCD.

The number of DCCD-binding sites in F_1-ATPase has been assayed by kinetic and binding assays. (1) Based on kinetic assays, the binding of one DCCD per active site of F_1-ATPase is sufficient to inactivate the enzyme to an extent of 100%. (2) On the other hand, direct binding assays with [^{14}C]DCCD have shown that 100% inactivation of F_1 activity coincides with the reaction of 2 mol of DCCD per mol of F_1-ATPase. Because of the strictly linear relationship between the binding of [^{14}C]DCCD and the ATPase inactivation, it is not possible to determine on kinetic grounds whether the two DCCD-binding groups are the same or different. However, glycine ethyl ester, a nucleophile which reacts selectively with carbodiimide-activated carboxyl groups (Figure 8) and which had no effect on the rate of inactivation of F_1-ATPase by DCCD, was found to prevent the incorporation of only one [^{14}C]DCCD per F_1-ATPase. Thus one group out of the two DCCD reactive groups in F_1-ATPase can be identified as carboxyl residue. It is not possible to specify whether ATPase inactivation is due to the binding of DCCD to this very carboxyl group. Indeed the possibility remains that the second DCCD reactive residue is also a carboxyl group but the rearrangement to give *N*-acylurea could be much faster than the reaction with glycine ethyl ester (Figure 8). Another plausible explanation of the discrepancy between binding of [^{14}C]DCCD and kinetic data on DCCD inactivation is that one of the two β subunits possesses a readily reactive carboxyl group, whereas the second carboxyl group in the second β subunit is not directly accessible to DCCD. Binding of DCCD to the first subunit would result in a 100% inactivation of the F_1-ATPase and in the same time in an unmasking of the second carboxyl group which then would become accessible to the binding of a second molecule of DCCD.

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Immunochemical Analysis of Membrane Vesicles from *Escherichia coli*†

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ABSTRACT: Membrane vesicles isolated from *Escherichia coli* ML 308-225 have been analyzed by crossed immunoelectrophoresis, and immunoprecipitates corresponding to the following cellular components have been identified: ATPase (EC 3.6.1.3), two or three NADH dehydrogenases (EC 1.6.99.3), D-lactate dehydrogenase (EC 1.1.1.27), glutamate dehydrogenase (EC 1.4.1.4), dihydro-orotate dehydrogenase (EC 1.3.3.1), 6-phosphogluconate dehydrogenase (EC 1.1.1.43), polynucleotide phosphorylase (EC 2.3.7.8), β-galactosidase (EC 3.2.1.23), lipopolysaccharide, and Braun's lipoprotein. The cellular origin of many of the vesicle immunogens is determined, and Braun's lipoprotein is used as a marker to quantitate the extent of outer membrane contamination (less than 3%). Membrane antigens are also characterized with regard to their amphiphilic or hydrophilic properties by charge-shift crossed immunoelectrophoresis. Furthermore, the following immunogens cross-react with components in membrane vesicles prepared from *Salmonella typhimurium*: one of the three NADH dehydrogenases, ATPase, polynucleotide phosphorylase, 6-phosphogluconate dehydrogenase, Braun's lipoprotein, and three unidentified antigens. In the accompanying paper [Owen, P., & Kaback, H. R. (1979) *Biochemistry 18* (following paper in this issue)] quantitative immunoadsorption is utilized to establish the topology of the vesicles with respect to the distribution of antigens on the inner and outer faces of the membrane.

Bacterial membrane vesicles have provided an increasingly important model system for the study of active transport (Kaback, 1972, 1974, 1976), and it is now apparent that they catalyze the accumulation of many solutes by a respiration-dependent mechanism in which chemiosmotic phenomena (Mitchell, 1961, 1966a,b, 1968, 1973) play a central, obligatory role (Schuldiner & Kaback, 1975; Kaback, 1976; Ramos et al., 1976; Ramos & Kaback, 1977a–c; Tokuda & Kaback, 1977). Although various lines of evidence indicate strongly that vesicles prepared by osmotic lysis (Kaback, 1971; Short et al., 1975) consist of topologically sealed plasma membranes with the same polarity as the intact cell (see Stroobant & Kaback, 1975, for a review), doubts have been expressed about the chemical nature and orientation of the preparations (Harold, 1972; Mitchell, 1973; Van Thienen & Postma, 1973; Hare et al., 1974; Futai, 1974; Weiner, 1974; Futai & Tanaka, 1975; Wickner, 1976; Adler & Rosen, 1977; Yamoto et al., 1978). Obviously, resolution of this controversy is of fundamental importance to the interpretation of results obtained from transport experiments conducted with this model system.

Recent studies demonstrate that crossed immunoelectrophoresis (CIE)[1] is an extremely powerful tool for the analysis of membrane immunogens (Johansson & Hjertén, 1974; Owen & Salton, 1975, 1977; McLaughlin & Meerovitch, 1975; Smyth et al., 1976, 1978; Alexander & Kenny, 1977, 1978). Not only does this two-dimensional immunoelectrophoretic technique resolve a spectrum of membrane components to a level approximating that of sodium dodecyl sulfate–polyacrylamide electrophoresis (Owen & Salton, 1975; Smyth et al., 1978) but membrane antigens analyzed by this method often retain sufficient biological activity to permit functional characterization (Owen & Smyth, 1977). Moreover, the quantitative aspects of the method (Weeke, 1973) allow the removal of antibodies during adsorption to be monitored with ease. Thus, expression of cell surface antigens as well as their distribution across the membrane can be established following adsorption of antiserum with whole cells, protoplasts, or isolated membranes (Johansson & Hjertén, 1974; Owen & Salton, 1975; Salton & Owen, 1976; Owen & Kaback, 1978).

Accordingly, we have undertaken a comprehensive immunochemical study of membrane vesicles prepared from *Escherichia coli* ML 308-225, and a preliminary report on some of the results has been presented (Owen & Kaback, 1978). In this paper, many of the antigens comprising the CIE reference pattern for solubilized ML 308-225 vesicles are characterized in detail with respect to their chemical nature, catalytic activity, amphiphilic or hydrophilic properties, and probable cellular origin. In addition, the cross-reactivity of certain ML 308-225 membrane immunogens with components in *Salmonella typhimurium* membrane vesicles is demonstrated.

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[1] Abbreviations used: CIE, crossed immunoelectrophoresis; $NaDodSO_4$ gel electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CTAB, cetyltrimethylammonium bromide; DOC, sodium deoxycholate; EDTA, ethylenediaminetetraacetic acid.

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