Dicyclohexylcarbodiimide Binds Specifically and Covalently to Cytochrome c Oxidase While Inhibiting Its H⁺-translocating Activity*

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We have investigated the covalent binding of dicyclohexylcarbodiimide (DCCD) to cytochrome c oxidase in relation to its inhibition of ferrocyanochrome c-induced H⁺ translocation by the enzyme reconstituted in lipid vesicles. DCCD bound to the reconstituted oxidase in a time- and concentration-dependent manner which appeared to correlate with its inhibition of H⁺ translocation. In both reconstituted vesicles and intact beef heart mitochondria, the DCCD-binding site was located in subunit III of the oxidase. The apolar nature of DCCD and relatively minor effects of the hydrophilic carbodiimide, 1-ethyl-(3-dimethylaminopropyl)-carbodiimide, on H⁺ translocation by the oxidase indicates that the site of action of DCCD is hydrophobic. DCCD also bound to isolated cytochrome c oxidase, though in this case subunits III and IV were labeled. The maximal overall stoichiometries of DCCD molecules bound per cytochrome c oxidase molecule were 1 and 1.6 for the reconstituted and isolated enzymes, respectively. These findings point to subunit III of cytochrome c oxidase having an important role in H⁺ translocation by the enzyme and indicate that DCCD may prove a useful tool in elucidating the mechanism of H⁺ pumping.

It has been proposed recently that cytochrome c oxidase (EC 1.9.3.1), the terminal electron-transfering enzyme of the mitochondrial respiratory chain, possesses an H⁺-translocating activity linked to its oxidation-reduction function (1-3). In our investigations of the cytochrome c oxidase H⁺ pump we have chosen as an experimental system lipid vesicles formed in the presence of the purified enzyme (4, 5). These reconstituted cytochrome c oxidase vesicles are well defined in terms of their composition and internal medium. As a result, the observations of H⁺ extrusion in this system are not open to many of the alternative interpretations which have been applied to experiments designed to demonstrate H⁺ pumping via cytochrome c oxidase in intact mitochondria (see Refs. 6-8). The initial observations of ferrocyanochrome c-induced H⁺ extrusion from such reconstituted vesicles by Wikström and co-workers (2, 9) have been verified in our laboratory, and it has been confirmed that the protons appearing on ferrocyanochrome c oxidation are indeed the result of translocation and not a result of the addition, binding, or oxidation of ferrocyanochrome c (10-12).

We have recently reported that N,N'-dicyclohexylcarbodiimide inhibits H⁺ translocation in reconstituted cytochrome c oxidase vesicles and mitochondria (13). This substance is a well established inhibitor of H⁺ translocation in ATP-linked systems (4-16) where it acts through specific, covalent binding to one hydrophobic subunit of the enzymes (17, 18). We were prompted, consequently, to investigate the possibility that DCCD may interact covalently with cytochrome c oxidase. We report here that DCCD does indeed exhibit a specific covalent binding to the oxidase and that the binding of DCCD appears to be correlated with its inhibition of the proton-translocating activity of the enzyme.

EXPERIMENTAL PROCEDURES

Preparative Methods

Beef heart mitochondria were prepared as described by Blair (19). Cytochrome c oxidase was purified according to the method of Yu et al. (20) and reconstituted in lipid vesicles as described elsewhere (10) except that 0.5 mM Hepes was used in the final dialysis to decrease the amount of buffer present in the proton translocation measurements.

Measurement of Proton Translocation by Cytochrome c Oxidase

Spectrophotometric Procedure—The absorbance of the experimental samples (composition as described in the figure legends) was monitored at 13°C and 566.5 - 504.5 nm using an Aminco DW-2a spectrophotometer. The pH was adjusted to 7.4, and sufficient ferrocyanochrome c for one turnover of the enzyme molecules was added. The resulting pH changes were calibrated using Merck Titrisol grade HCl.

Potentiometric Procedure—The media and additions were as for the spectrophotometric procedure except that no phenol red was present. The apparatus was as described previously (10).

Binding of [¹⁴C]DCCD to Cytochrome c Oxidase

Cytochrome c oxidase, either free or reconstituted into vesicles, was incubated with [¹⁴C]DCCD as described in the figure legends. To terminate the incubations, 5-mL aliquots of an ice-cold chloroform/methanol mixture (1:4, v/v) were added. The samples were immediately centrifuged at 4000 rpm for 5 min on a bench centrifuge. The supernatants were discarded, and a further 5 ml of the mixture was added. Following mixing, the samples were centrifuged again, and the pellets were dispersed in 0.4 mL of 25 mm Tris acetate buffer, 3% NaDodSO₄, 2 mM EDTA, pH 8.2, under gentle sonication. Some of this dispersion (0.2 mL) was added to 7 mL of the liquid scintillation mixture Lipoluma, Lumasolve (Lumac System AG, Basel), water (50:5:1, v/v) and the radioactivity measured. Further samples of the dispersion were assayed for protein content according to the method of Lowry et al. (21).

Determination of the [¹⁴C]DCCD-binding Subunit(s)

Cytochrome c oxidase was incubated with [¹⁴C]DCCD as described...

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in the legend to Fig. 6. Samples (0.4 ml each) of the suspensions were then added to 50 ml of 20% potassium chloride and layered onto 3.2 ml of 10% sucrose. They were then centrifuged for 2 h at 4°C and 175,000 g_{\text{max}} to 425,000 g_{\text{max}} in the swing-out rotor (6 × 4.2 ml) of an MSE Prepspin 65 centrifuge. The resulting pellets were dissolved in 30 ml of 0.2 M Tris acetate, 2 mM EDTA, 3% NaDodSO_{4}, and then underwent electrophoresis on 12% polyacrylamide gels in 0.35 M Tris acetate, 0.5% NaDodSO_{4}, pH 8.2. The gels were stained and destained as described by Weber and Osborn (22). They were then scanned at 600 nm using a Gelman ACD-15 gel scanner. Following dialysis for 40 h versus 7% CH_{3}COOH, 10% ethanol the gels were sliced, and the slices were heated for 5 h at 80°C with 0.2 ml of 30% H_{2}O_{2}, 1% concentrated ammonia solution and then assayed for radioactivity using 7 ml of the scintillation fluid described above.

Fluorescence was measured using a Perkin-Elmer MPF-2a fluorescence spectrophotometer fitted with a thermostatted cuvette holder.

**Reagents**

Cytochrome c (horse heart, type V1) from Sigma Chemical Co. was converted to the ferrous form as described elsewhere (10). Soybean phospholipids from Sigma Chemical Co. were recrystallized. Valinomycin, CCCP, and EDAC were from Sigma Chemical Co. 9-Amino acridine was from Aldrich. N,N’-Dicyclohexylcarbodiimide was from Fluka AG, Buchs, Switzerland, and N,N’-dicyclohexyl[14C]carbodiimide was from CEA, Gif-Sur-Yvette, France. Other reagents were of analytical grade.

**RESULTS**

(i) **The Inhibition of H\(^+\) Extrusion from Reconstituted Cytochrome c Oxidase Vesicles by DCCD is not a Protonophorusr Effect**—DCCD inhibits H\(^+\) translocation via cytochrome c oxidase in reconstituted vesicles (13). It is very important to establish that this inhibition does not occur simply through disruption of the vesicular membranes, thus increasing their H\(^+\) permeability. In this case, the rate of decay of the ferrocytochrome c-induced H\(^+\) pulse might be increased and consequently the size of the pulse underestimated (10). To exclude this possibility, the effect of DCCD on the H\(^+\) permeability of the reconstituted vesicles was measured directly, in parallel with its inhibition of the ferrocytochrome c-induced H\(^+\) extrusion. Similar measurements were carried out using the protonophore CCCP. The change in H\(^+\) permeability was measured by following the rate of buildup of a pH gradient across the vesicular membranes induced by a transmembrane K\(^+\) gradient in the presence of valinomycin. The ΔpH was monitored using 9-amino acridine, whose fluorescence in the presence of lipid vesicles is quenched on the induction of a pH gradient, inside acid, across the vesicular membranes (23, 24). As shown in Fig. 1, the inhibition of ferrocytochrome c-induced H\(^+\) extrusion by DCCD was accompanied by extremely small changes in the H\(^+\) permeability, whereas CCCP clearly diminished the size of the pulse of extruded protons through a protonophoric effect. These observations were corroborated by the much slower decays of the pulses obtained following treatment of the vesicles with DCCD compared to those in the presence of CCCP (not shown). The above results indicate that DCCD cannot exert its inhibitory action on the cytochrome c oxidase H\(^+\) pump by increasing the membrane H\(^+\) permeability.

It is noteworthy that, owing to the relatively high stability of the vesicles at 13°C, it was possible to incubate them with DCCD at this temperature for longer times than reported previously (13) leading to a more potent inhibition.

(ii) **Comparison of the Effects of DCCD and of a Hydrophilic Carbodiimide on the Cytochrome c Oxidase H\(^+\) Pump**—To determine the importance of the hydrophobicity of DCCD on the inhibition of H\(^+\) translocation, the effect of a hydrophilic carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was also investigated (Table I). At concentrations at which the ferrocytochrome c-induced H\(^+\) extrusion from reconstituted vesicles was strongly inhibited by DCCD, EDAC had only relatively minor effects.

(iii) **Binding of DCCD to Cytochrome c Oxidase**—It was of interest to investigate the possibility that the inhibition of the cytochrome c oxidase proton pump by DCCD is linked to specific covalent binding of DCCD to the enzyme, as occurs in H\(^-\) (17, 18) and possibly Ca\(^2+\) (25) translocating ATPases. As shown in Fig. 2, [\(^{14}\)C]DCCD did indeed bind to the oxidase, and the time course of binding was remarkably similar to that of inhibition of the H\(^+\) pump by DCCD. Fig. 2 also shows that during the incubation with DCCD there was no increase in the buffering capacity of the system (if anything a slight decrease occurred). This has important consequences for the validity of the DCCD inhibition as discussed below. Comparison of the curves of Fig. 2 indicates that between 0.75 and 1 molecule of oxidase was inhibited in its H\(^+\)-translocating activity per molecule of DCCD bound. The extent of binding showed remarkable reproducibility among different preparations of vesicles. For example, incubation of six different vesicle preparations with DCCD at a concentration of 70 mol per mol of oxidase for 3 h at pH 6.8 and 13°C gave a mean of 0.210 mol of DCCD bound per mol of oxidase with a standard deviation of 0.019.

The binding of [\(^{14}\)C]DCCD to the reconstituted enzyme was
Table 1

Comparison of the effects of DCCD and EDAC on H⁺ translocation in cytochrome c oxidase vesicles

| Moles of carbodiimide per mol of enzyme | Inhibition of H⁺ translocation in samples incubated with |
|----------------------------------------|--------------------------------------------------------|
|                                        | DCCD | EDAC |
| 102                                    | 69.5 | 11.5 |
| 205                                    | 78.5 | 23.0 |
| 512.5                                  | 79.7 | 28.2 |

Fig. 2. The time dependence of DCCD binding to cytochrome c oxidase in reconstituted vesicles and its inhibition of proton translocation. 

- 3A: 0.5 ml of cytochrome c oxidase vesicles (3.1 nmol of enzyme) was diluted with 0.5 ml of 79 mM sucrose, 25 mM KCl, 50 μM phenol red, and pH 6.8. 25 mM DCCD (100 mM, 0.5 Ci per mol) were added. The samples were incubated at 13°C, and at the times indicated the incubations were terminated, lipid and unbound DCCD removed, and protein content and radioactivity determined as described under "Experimental Procedures." The inhibition of activity of the DCCD-containing samples is shown as a percentage with respect to the corresponding ethanol control. The buffering capacities of the samples were measured using standard pulses of HCl immediately after determination of the H⁺ translocating activity and are shown as the mean ± S.D. of three determinations.

- 3B: The plots of bound versus added DCCD gave the lines shown in Fig. 3B when plotted in a double reciprocal manner. When these curves were analyzed by the curve-fitting method described in the legend to Fig. 3B, it was found that they intercepted the y axis at the point shown. The reciprocal of this value indicated that the number of moles of DCCD bound per mol of oxidase at infinite DCCD concentration was 1.

The binding of [³⁵Cl]DCCD increased slightly with the pH at which the incubation was performed (Fig. 4). The pH dependencies were very similar in the presence of valinomycin and CCCP, when the intra- and extravesicular pH values should have been identical, and in the absence of these ionophores when the internal pH should have remained fairly constant and only the external pH varied.

[³⁵Cl]DCCD also bound in a time-dependent manner to free cytochrome c oxidase in the presence of a small amount of detergent to stabilize the enzyme in solution. The concentration dependence of the binding is shown in Fig. 5. Unlike the case for the reconstituted oxidase a double reciprocal plot of the data gives a straight line whose intercept on the x axis

Fig. 3. The concentration dependence of DCCD binding to cytochrome c oxidase in reconstituted vesicles. A. 0.5 ml of cytochrome c oxidase vesicles (2.98 to 3.15 nmol of enzyme, 20 mg of lipid) was diluted with 0.5 ml of 1 mM MES, 30 mM KCl, 79 mM sucrose, pH 6.8, followed by the addition of [³⁵Cl]DCCD to give the concentrations indicated, the ethanol content being maintained constant in all samples. The suspensions were incubated at 13°C, and after 40 (●), 90 (●), or 180 (○) min the incubations were terminated, lipid and unbound DCCD removed, and protein content and radioactivity determined as described under "Experimental Procedures." B, a double reciprocal plot of the data of A. The intercept shown gives the best least squares fit to a power curve (correlation coefficients, 0.995, 0.998, and 0.997 for the 40 (●), 90 (●), and 180 (○) min curves, respectively) of all intercepts tested between 0 and 1.5.
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Fig. 4. pH dependence of DCCD binding to reconstituted cytochrome c oxidase. 0.5-ml aliquots of cytochrome c oxidase vesicles (3.6 or 4.15 nmol of enzyme, 20 mg of lipid) were added to 1-ml aliquots of 73 mM sucrose, 27 mM KCl containing the following buffers at 10 mM concentrations: acetate (pH 5 and 5.5), MES (pH 6 and 6.5), Hepes (pH 7 and 7.5), or Tris (pH 8 and 8.5). KOH was used to adjust the pH of all solutions except Tris, where HCl was used. The suspensions were incubated at 13°C following the addition of [14C]DCCD (100 mM, 0.5 Ci/mol) to give a final concentration of 70 mol/mol of enzyme. To each of one set of samples (A) was added 20 µl of 10 mM CCCP and 2 µl of 2 mM valinomycin and to another set (B) there were no further additions. After 3 h the incubations were terminated, lipid and unbound DCCD removed, and protein content and radioactivity determined as described under "Experimental Procedures." Indicating a maximal binding of 1.6 nmol of DCCD/nmol of cytochrome c oxidase.

(iv) The Site of Binding of DCCD to Cytochrome c Oxidase—The binding of DCCD to both reconstituted and free cytochrome c oxidase appears to have been covalent as the label was not removed by washing with an apolar solvent system. It was important to establish whether the binding occurs only at a certain region of the oxidase or is not site specific. In investigating this question it was not possible to employ the procedure used in the binding experiments mentioned above, as treatment of the oxidase with the chloroform/methanol mixture used, for aggregation of the high molecular weight subunits resulting in an abnormal pattern in NaDodSO4 polyacrylamide gel electrophoresis. Consequently, in order to remove lipid and unbound DCCD following incubation of the oxidase with [14C]DCCD, a centrifugation procedure, followed by extensive washing, was employed, as described under "Experimental Procedures." Fig. 6 shows the NaDodSO4 polyacrylamide gel electrophoresis pattern obtained from cytochrome c oxidase incubated with [14C]DCCD when reconstituted into vesicles (A) and when free (B). In the case of the reconstituted enzyme, subunit III alone was labeled, and this was found reproducibly in five different enzyme preparations. With the free enzyme, [14C]DCCD bound to both subunits III and IV to approximately similar extents. Similar extents of binding of [14C]DCCD with time were obtained with the washing procedure used in the electrophoresis studies and that using the chloroform/methanol mixture (not shown) indicating that the mode of binding was the same in both types of experiment.

For the binding of DCCD to cytochrome c oxidase to be physiologically relevant, it is important to establish that it also occurs in the intact mitochondrial system. As shown in Fig. 7, when beef heart mitochondria were incubated with [14C]DCCD and the cytochrome c oxidase extracted and analyzed, subunit III alone was again labeled as in reconstituted vesicles. Owing to the necessity of using a relatively small amount of starting material, it was not possible to obtain a totally purified preparation of cytochrome c oxidase from the

Fig. 5. The concentration dependence of DCCD binding to nonreconstituted cytochrome c oxidase. 100 µl of 360 µM cytochrome c oxidase was added to 3.2 ml of 0.5% Tween, 5 mM MES, pH 6.8. [14C]DCCD was added to 0.5-ml aliquots of this mixture to give the concentrations indicated, the ethanol content being kept constant. The samples were incubated at 13°C for 3 h, and then the incubations were terminated and samples treated as described for Fig. 2. The inset shows the results presented in the form of a double reciprocal plot. The data was fitted to a straight line using a linear regression program giving a correlation coefficient of 0.996.

Fig. 6. DCCD-binding subunits in reconstituted and free cytochrome c oxidase. 0.5 ml of cytochrome c oxidase vesicles in 30 mM KCl, 79 mM sucrose, 1 mM Hepes, pH 7.4, containing 4.27 nmol of enzyme (A) or 50 µl of 250 mM sucrose, 50 mM sodium phosphate, pH 7.4, containing 1.38 nmol of enzyme (B) was added 8.5 µl or 2.8 µl, respectively, of [14C]DCCD (10 mM, 50 Ci/mmol). The mixtures were incubated at 4°C for 15 h, and then lipid and unbound DCCD were removed, and the resulting enzyme samples analyzed as described under "Experimental Procedures." The smooth line shows the distribution of protein and the other line the distribution of radioactivity on the subsequent NaDodSO4 polyacrylamide gel electrophoresis.
mitochondria. The purification procedure was terminated after preparation of the fraction which Yu et al. (20) have referred to as the crude cytochrome c oxidase fraction. This was contaminated with what were probably core proteins of cytochrome b(568) complex (20) which appear as the high molecular weight bands in Fig. 7. These proteins were clearly not labeled by [14C]DCCD. Identification of the subunits of cytochrome c oxidase was carried out by comparison with the pattern obtained from a gel electrophoresis carried out under conditions identical with those of Fig. 7, on a preparation of cytochrome c oxidase purified in the normal way (20). It is, of course, not possible to derive a stoichiometry of binding of DCCD in the intact mitochondrial system as other DCCD-binding proteins were present.

Effects of Dicyclohexylcarbodiimide on Ferrocytochrome c Oxidation by Reconstituted Vesicles—We have reported previously (13) that the inhibition of the cytochrome c oxidase H⁺ pump by DCCD is accompanied by only a relatively minor inhibition of the rate of oxidation. Table II shows that following longer incubation of reconstituted vesicles with DCCD, giving more potent inhibition of H⁺ translocation than was observed previously (see Table I), there was still only a mild inhibition of ferrocytochrome c oxidation. DCCD also caused a small inhibition of oxidation in vesicles incubated in the presence of CCCP and of CCCP plus valinomycin, and also in the isolated oxidase (Table II).

**D I S C U S S I O N**

Interaction of DCCD with Reconstituted Cytochrome c Oxidase—It is essential to establish that the inhibition by DCCD of H⁺ translocation in reconstituted cytochrome c oxidase vesicles (13) represents a real effect of DCCD on the enzyme and not an interaction with some other component of the experimental system. As shown in Fig. I the inhibitory effects of DCCD are not due to an increase in membrane H⁺ permeability of the kind displayed by 2-n-heptyl-4-hydroxyquinoline N-oxide at higher concentrations in mitochondria (27). As shown in Fig. 2, there was also no increase of available buffering power of the vesicular suspension concomitant with the inhibition by DCCD. Such an increase should have occurred had the membranes become leaky to H⁺, much of the buffering power being located at the vesicular interior (10). Furthermore, if DCCD did exert a protonophorous effect, then, under conditions where H⁺ translocation was inhibited, the rate of cytochrome c oxidation should have been stimulated, as shown in Table II, there was in fact a mild inhibition of oxidation by DCCD.

The decrease in ferrocytochrome c-induced H⁺ extrusion following incubation with DCCD was not simply an apparent effect, owing to a DCCD-induced change in the pH dependence of phenol red absorbance. Addition of standard pulses of HCl before and after incubation with DCCD for sufficient time to afford inhibition caused the same changes in the A504,5-504,5 (see Fig. 2). Moreover, effects of DCCD similar to those detected with phenol red were observed using a glass electrode to detect the pH changes; this latter method was not used routinely, however, owing to its slow response time, relative to that of the phenol red system.

The effects of DCCD were not due to its interaction with valinomycin, thus preventing the transmembrane K⁺ move-

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**Table II**

The effects of DCCD on oxidation of ferrocytochrome c by isolated and reconstituted cytochrome c oxidase

| Vesicles | Moles of DCCD/DCCD | Oxidative activity (molecules of cytochrome c per s per mol of oxidase) |
|----------|-------------------|-----------------------------|
| 0        | 12.9              | 6.2                         |
| 102      | 12.1              | 12.4                        |
| 205      | 11.3              | 21.7                        |
| 512.5    | 10.1              | 22.5                        |
| Vesicles + CCCP | 0               | 23.6                        |
| 512.5    | 18.3              | 22.5                        |
| Vesicles + CCCP + valinomycin | 0               | 45.0                        |
| 512.5    | 36.0              | 20.0                        |
| Isolated enzyme | 0               | 40.1                        |
| 102      | 38.4              | 4.3                         |
| 205      | 38.8              | 3.2                         |
| 512.5    | 38.5              | 4.0                         |
DCCD Binding to Cytochrome c Oxidase

ment which is essential for the detection of the ferrocytochrome c-induced H⁺ efflux (1). The inhibition increased with increased time of incubation with DCCD (Figs. 1 and 2) while valinomycin was always present only for the final 5 min of these incubations. Also, no effects were observed of DCCD on the rate of K⁺/H⁺ exchange in reconstituted vesicles in the presence of CCCP.

On the basis of the above arguments, we conclude that the inhibition by DCCD of H⁺ translocation by cytochrome c oxidase is indeed a genuine effect of this substance on the enzyme.

The covalent binding of DCCD to cytochrome c oxidase in a time- and concentration-dependent manner is, we feel, a particularly interesting observation. In reconstituted vesicles the binding of DCCD is apparently linked to its inhibitory effect on the H⁺ pump as indicated by the similar time courses of the two. There is an approximately 1:1 relationship between the proportion of oxidase molecules labeled by DCCD and the proportion of H⁺-translocating activity which was inhibited (Fig. 2). This, together with the observation that maximally 1 molecule of DCCD was bound per cytochrome c oxidase molecule (Fig. 3) is consistent with there being one specific binding site for DCCD per cytochrome c oxidase molecule which plays a central role in H⁺ translocation by the enzyme.

The observation that DCCD binds exclusively to subunit III of the oxidase in reconstituted vesicles (Fig. 6A) and also in intact mitochondria (Fig. 7) points, therefore, to this subunit being an important part of the cytochrome c oxidase H⁺ pump.

A central role for subunit III of cytochrome c oxidase in transmembrane H⁺ movements is particularly feasible considering that it is very hydrophobic in its amino acid composition (28, 29) and that there is evidence that it spans completely the mitochondrial membrane (30). Further support for this subunit forming at least part of the link between electron flow and H⁺ translocation in cytochrome c oxidase comes from recent observations of Penttilä et al. (31). They found that a preparation of cytochrome c oxidase which was totally depleted of subunit III had twice the activity of the original preparation.

This would be consistent with subunit III being the site of coupling between oxidation and H⁺ translocation, whereby its removal would decrease the "load" on cytochrome c oxidation by the enzyme and consequently increase its rate.

EDAC is a highly reactive, hydrophobic carbodiimide which interacts covalently with several proteins (32-35) including the H⁺ pump of the Halobacterium halobium purple membrane which it also inhibits (35). The fact that the inhibitory effects of EDAC on the cytochrome c oxidase H⁺ pump were very small in comparison to those of DCCD may indicate, therefore, that the binding site of the latter within subunit III is hydrophobic in nature.

It is of interest to consider which amino acids of subunit III might interact covalently with DCCD. Carbodiimides are extremely reactive substances (36) but have been shown to interact covalently with only four kinds of amino acids in proteins. Hydrophobic carbodiimides form covalent adducts with tyrosine residues in chymotrypsinogen (32) and yeast hexokinase (34), inhibiting the latter, and with cysteine residues in β-lactoglobulin (33). The effects of DCCD in inhibiting H⁺-translocating ATPases are due to its covalent interaction with glutamic acid residues in Neurospora crassa and Saccharomyces cerevisiae and aspartic acid in Escherichia coli (37). From amino acid composition studies (28, 29) it appears that the only three amino acids present in subunit III of cytochrome c oxidase are glutamic acid (approximately 9%), aspartic acid (approximately 6.5%), and tyrosine (approximately 4%).

DCCD binding increased with increasing pH of incubation. The fact that similar pH effects on binding were observed in the presence and absence of CCCP plus valinomycin indicates that the effects were exerted at the external side of the membrane. As the binding probably took place at a hydrophobic site within the enzyme the pH of the medium is unlikely to have had any effect on the degree of protonation of the group involved. It is more likely that a pH-induced change in the conformation of the oxidase allowed easier access of DCCD to its binding site or more favorable conditions for binding, or both.

The results of Table II, cf. those of Table I, confirm our earlier findings that DCCD has only minor effects on the oxidative activity of reconstituted cytochrome c oxidase, which appears inconsistent with its inhibitory effects on H⁺ translocation. We feel, however, that lack of detection of the inhibition may be explained by the effects of the inhibitory H⁺ gradient, built up on cytochrome c oxidase and by removal from the vesicular interior of H⁺, a co-substrate in the reduction of O₂ by cytochrome c. In the coupled system these inhibitory effects should be built up very rapidly, considering the low intravesicular volume (38) and thus, in the steady state oxidation rates reported here, further inhibitory effects of DCCD would be masked. In the presence of a protonophore, the oxidative activity was not stimulated maximally as revealed by its further stimulation by valinomycin as found elsewhere (39). This shows that, even in the presence of CCCP, the vesicular membranes had a finite resistance to H⁺ movement which indicates that even under these conditions, substrate (i.e. H⁺) deficiency could continue. In this case the further inhibitory effects of DCCD would again be reduced.

An alternative explanation of this data is that DCCD causes the H⁺ consumed in O₂ reduction to be taken from the extrasomal instead of the intravesicular space. This would lead to a decrease in the net H⁺ extrusion but might have only a minor effect on oxidation.

Interaction of DCCD with Isolated Cytochrome c Oxidase—[¹⁴C]DCCD also bound in a time- and concentration-dependent manner to isolated cytochrome c oxidase. Unlike the case for the reconstituted enzyme, however, with the isolated oxidase, both subunits III and IV were labeled (Fig. 6B). This would explain the higher maximal stoichiometry of DCCD binding obtained with the isolated, compared to the reconstituted, enzyme. Subunit IV of cytochrome c oxidase does not have a particularly hydrophobic amino acid composition (29) and protrudes into the bulk phase from the inner face of the inner mitochondrial membrane (30). This would clearly not favor the stable binding of DCCD which requires a hydrophobic environment (36) and explains why no labeling of subunit IV by DCCD was observed with cytochrome c oxidase in mitochondria (Fig. 7) or in reconstituted vesicles (Fig. 6A). The lipid-free cytochrome c oxidase used here forms aggregates in aqueous suspension, and under these conditions subunit IV could be forced into a more hydrophobic environment, more favorable to DCCD binding.

DCCD interacts specifically and covalently with cytochrome c oxidase concomitantly with its inhibition of H⁺ translocation by the enzyme. We conclude that DCCD, with its well defined chemistry, promises to be a useful tool in the investigation of this oxidation-reduction-linked H⁺ pump.

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