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Studies on the Molecular Basis of H^+ Translocation by Cytochrome *c* Oxidase[1]

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Abstract

We report here studies which characterize further the interaction of *N,N'*-dicyclohexylcarbodiimide with cytochrome *c* oxidase leading to inhibition of H^+ translocation by the enzyme. Further evidence is presented to show that the inhibition results from a real interaction of DCCD with the enzyme and cannot be accounted for by uncoupling and, contrary to recent criticisms, this interaction occurs specifically with subunit III of the enzyme even at relatively high inhibitor-to-enzyme stoichiometries. Use of a spin-label analogue of DCCD has enabled us to demonstrate that the carbodiimide-binding site is highly apolar and may not lie on the pathway of electron transfer.

Key Words: cytochrome; oxidase; mitochondria; structure membrane; protein; subunits; oxidation-reduction.

Introduction

The H^+ translocating function of mitochondrial cytochrome *c* oxidase, as proposed by Wikström and his co-workers (Wikström, 1977; Wikström and Saari, 1977; Wikström and Krab, 1979), has been the subject of considerable attention recently.

While evidence has been presented against this deviation from the classical view of the enzyme's role in chemiosmotic coupling (Moyle and Mitchell, 1978a; Moyle and Mitchell, 1978b; Lorusso *et al.*, 1979; Papa *et al.*, 1980), there seems to be a good case in favor of the oxidase indeed functioning as a redox-linked proton pump (Sorgato and Ferguson, 1978;

[1]Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; NCCD, *N*-(2, 2, 6, 6-tetramethylpiperidyl-1-oxyl)-*N'*-(cyclohexyl)carbodiimide; Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl) ethane sulfonate; TMPD, *N,N,N',N'*-tetramethylphenylenediamine.

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Casey *et al.*, 1979; Sigel and Carafoli, 1978; Sigel and Carafoli, 1979). At this point, it is opportune to consider how the energy made available on oxidation of cytochrome *c* might be coupled to the movement of protons against a barrier of electrochemical potential difference. As this coupling probably involves the complex subunit structure of the enzyme, it is of interest to investigate the involvement of any particular subunit or subunits in H^+ pumping activity.

Subunit II appers to be the site of cytochrome *c* (Bisson *et al.*, 1978) and copper (Winter *et al.*, 1980) binding in the oxidase, whereas the hemes are probably located in subunits I and II (Winter *et al.*, 1980). Our recent proposal (Casey *et al.*, 1980b) that subunit III of cytochrome *c* has an important role in H^+ translocation, therefore, could implicate this subunit directly in the coupling of oxidation to H^+ movement.

We report here further studies carried out to establish the involvement of subunit III in H^+ pumping using the inhibitor dicyclohexylcarbodiimide, and some indications of the proximity of the DCCD binding site to the electron transfer pathway, obtained using a spin-label analogue of DCCD.

Materials and Methods

Bovine heart cytochrome *c* oxidase was prepared according to the method of Yu *et al.* 1975) and used to prepare reconstituted vesicles as described elsewhere (Casey et al., 1979).

Ferrocytochrome *c*-induced H^+ extrusion from these reconstituted vesicles was measured spectrophotometrically. The absorbance of the samples of reconstituted vesicles in 25 mM KCl, 75 mM choline chloride, 50 μM Phenol Red, pH 7.4, was monitored at 13°C and 556.5–504.5 nm using an Aminco DW-2a spectrophotometer. Valinomycin (2.5 μl, 0.2 mM) was added and, when the pH had stabilized, sufficient ferrocytochrome *c* for one turnover of the oxidase molecules was added.

Following incubation with radioactive DCCD, samples of the suspensions were centrifuged at 175,000 g_{min} to 425,000 g_{max} for 2.5 hr at 4°C. The resulting pellets were electrophoresed and the gels were stained and destained according to the method of Weber and Osborn (1969). Following scanning and dialysis versus 7% acetic acid and 10% ethanol, the gels were sliced and assayed for radioactivity.

Oxygen reducing activity was measured by adding 10 μl of the reconstituted vesicles (0.062 nmol of heme) to 3.3 ml of 40 μM cytochrome *c*, 30 mM sodium ascorbate, 20 mM Hepes, 91 μM TMPD, pH 7.4, in the cuvette of a Clark-type oxygen electrode at 25°C. For measurement of the coupled rate of O_2 reduction there were no further additions. For the uncoupled rate 2.5 μl of 0.2 mM valinomycin and 5 μl of 10 mM CCCP were added.

For labeling with NCCD, cytochrome *c* oxidase vesicles (15 nmol of enzyme in 10 mM Hepes, 39.6 mM KCl, 50.4 mM sucrose, pH 7.4) or isolated cytochrome *c* oxidase (15 nmol in 0.5% Tween 80, 10 mM Hepes, pH 7.4) were incubated with 1.5 or 6 μmol of NCCD at 4°C for 22 hr. Similar results were obtained using both these amounts of spin label. To remove unincorporated NCCD, sodium cholate was added to the cytochrome *c* oxidase or vesicle suspensions to a concentration of 1.5% and ammonium sulfate was added until the enzyme precipitated. After collection by centrifugation, the enzyme was subjected to further similar washings. Removal of free spin label was facilitated by passage of the enzyme down a column of Sephadex G-25 equilibrated with 1.5% sodium cholate, 50 mM sodium phosphate, pH 7.4, and elution using the same solution.

ESR spectroscopy was carried out using a Varian E-104 Century Series spectrometer. Resonance of the nitroxide spin label was centered on 3380 G using a microwave frequency of 9.415 GHz. The frequency of modulation was 100 kHz and the spectrum plotting time was 8 min.

Ferrocytochrome *c* (horse heart, type VI) from Sigma Chemical Co. was prepared as described elsewhere (Casey *et al.*, 1979). Soybean phospholipids from Sigma Chemical Co. were purified further by three acetone precipitations from solution in ether.

Valinomycin and CCCP were from Sigma Chemical Co. *N,N'*-Dicyclohexylcarbodiimide was from Fluka AG, Buchs, Switzerland, and *N,N'*-dicyclohexyl [^{14}C]-carbodiimide was from CEA, Gif-Sur-Yvette, France. *N*-(2, 2, 6, 6-tetramethylpiperidyl-1-oxyl)-*N'*-(dyclohexyl) carbodiimide was prepared in this laboratory according to the method of Azzi *et al.* (1973).

Results

DCCD Inhibition of Cytochrome c Oxidase H^+ Pumping is not Due to Uncoupling

When ferrocytochrome *c* is added to a suspension of reconstituted cytochrome *c* oxidase vesicles with a high internal buffer concentration and in the presence of valinomycin and external potassium ions, a pulse of protons extruded via the oxidase is observed (Casey *et al.*, 1979; Sigel and Carafoli, 1979; Krab and Wikström, 1978). If the pulse of extruded protons is allowed to decay completely, a net consumption of one proton per electron passing to oxygen is observed (Casey *et al.*, 1979), as predicted by

$$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$$

A typical cytochrome *c*-induced proton pulse and its inhibition by DCCD are shown in Fig. 1 and Table I.

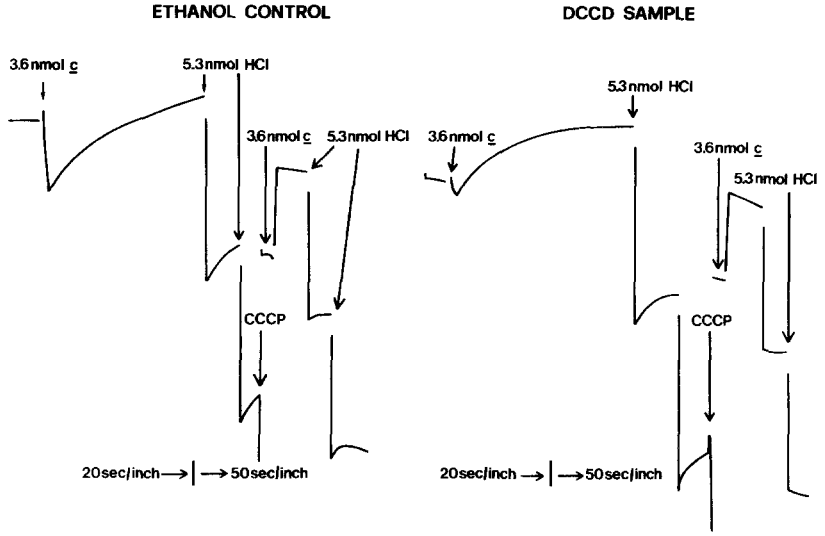

Fig. 1. Inhibition of H^+ translocation by DCCD is not an uncoupling phenomenon. Aliquots (0.1 ml) of reconstituted cytochrome *c* oxidase vesicles containing 0.54 nmol of the enzyme were added to 1.3 ml of 25 mM KCl, 75 mM-choline chloride, 50 μM Phenol Red, pH 7.4, followed by 11 μl of 20 mM DCCD in ethanol (to the DCCD sample) or 11 μl of ethanol (to the control sample). The suspensions were then incubated at 4°C for 22 hr and then the ferrocytochrome *c*-induced H^+ translocation was measured as described under "Material and Methods." The vertical discontinuities in the absorbance deflections represent corrections for artifacts due to addition of cytochrome *c* or HCl.

Following completion of H^+ extrusion, the pulse decays and it is clear that if the decay were sufficiently rapid the extent of H^+ extrusion would be underestimated (see also Casey *et al.*, 1979). Consequently, it is extremely important for the validity of the inhibition of H^+ extrusion by DCCD that a protonophorous or disruptive effect leading to acceleration of the H^+ pulse decay be excluded. The experiment of Fig. 1 carried out in the presence of DCCD shows that the diminution of H^+ extrusion is not due to an effect on the initial rate of pulse decay, which is practically the same as that obtained

Table I. Parameters of H^+ Translocation by Reconstituted Cytochrome *c* Oxidase Vesicles Under Control and DCCD-Inhibited Conditions[a]

	H^+ ejected (ng-ions per electron)	Initial rate of H^+ backflux (ng-ions/sec)	Buffering capacity in absence of CCCP (%)	H^+ consumed in presence of CCCP (ng-ions per electron)
Ethanol control	0.80	0.170	100	1.02
DCCD	0.19	0.110	98	1.01

[a]These values were calculated using the data from Fig. 1 after correction for baseline drift.

with the control sample (see Table I). Further evidence against a disruptive effect of DCCD on the vesicular membranes comes from the close similarity between the pH changes caused by the addition of 5.3 ng-eq of HCl to the DCCD sample and the ethanol control. As a considerable amount of the available buffering power lies inside the vesicles, one would expect a smaller pH change on adding the acid to the DCCD sample if DCCD caused the membranes to become leaky to protons.

Following addition of the protonophore CCCP to the samples, an immediate alkalinization occurred in both samples on addition of cytochrome *c*, corresponding to close to 1 H^+ consumed per electron passing to O_2.

Specific Labeling by DCCD of Cytochrome c Oxidase

While we have demonstrated a specific binding of DCCD to subunit III of cytochrome *c* oxidase reconstituted into vesicles under conditions similar to those used to obtain inhibition of the H^+ pump (Casey *et al.*, 1980b), the DCCD-to-enzyme stoichiometries used there were below those normally used for inhibition. The experiment of Fig. 2 shows that a similar binding of labeled DCCD to the enzyme occurs when a stoichiometry of 133 mol DCCD per mol enzyme is employed. Under these conditions clear inhibitions of H^+

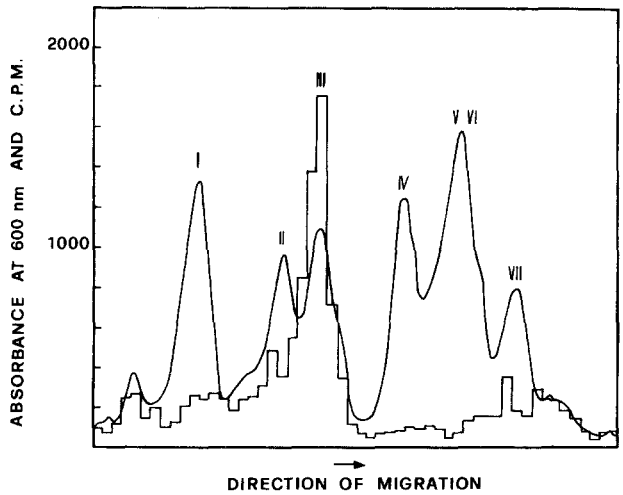

Fig. 2. Subunit analysis of DCCD-labeled cytochrome *c* oxidase. To 0.5 ml of reconstituted cytochrome *c* oxidase vesicles in 30 mM KCl, 79 mM sucrose, 1 mM Hepes, pH 7.4, containing 4.27 nmol of oxidase was added 568 nmol of ^{14}C-DCCD and the suspension was incubated at 4°C for 15 hr. Lipid and unbound DCCD were then removed and the resulting enzyme analyzed as described under "Materials and Methods." The smooth line shows the distribution of protein and the other line the distribution of radioactivity on the subsequent $NaDodSO_4$ polyacrylamide gel electrophoresis.

Table II. Inhibition of H^+ Translocation and Cytochrome *c* Oxidation in Cytochrome *c* Oxidase Vesicles by DCCD[a]

Sample	H^+ ejected (ng-ions per electron)	H^+ consumed in the presence of CCCP (ng-ions per electron)	Coupled rate of O_2 reduction (electrons passing to oxygen per second per oxidase molecule)	Uncoupled rate of O_2 reduction in the presence of CCCP and valinomycin (electrons passing to oxygen per second per oxidase molecule)
Control	0.79	0.95	51	311
DCCD-containing	0.32	0.98	38	220
Inhibition (%)	59.5	−3.2	25.6	29.3

[a]Samples (0.125 ml) of reconstituted cytochrome *c* oxidase vesicles (0.7 nmol heme) were added to 1.75 ml of 75 mM choline chloride, 25 mM KCl, 50 μM Phenol Red, pH 7.4. To each sample was added 10 μl of 9.3 mM DCCD in ethanol or 10 μl of ethanol. The samples were incubated for 15 hr at 4°C, and then their H^+-translocating and O_2-reducing activities were measured as described in the Materials and Methods section.

translocation and of cytochrome *c* oxidation were observed (see Table II), though, as found previously (Casey *et al.* 1980b), the inhibition of oxidation was lower than that of H^+ extrusion.

Spin-Label Studies on the Location of the DCCD Binding Site

In order to obtain further structural information on the region of DCCD binding within the oxidase, we employed NCCD, the spin-labeled analogue of DCCD. We have found that this substance binds covalently to cytochrome *c* oxidase (Casey *et al.*, 1980a) and the observations that pre-incubation with DCCD inhibits NCCD binding and that NCCD elicits a similar inhibition of H^+ translocation as does DCCD indicate that NCCD binds to the oxidase at the same site as does DCCD. In the experiment of Table III various reductants were added to NCCD-labeled cytochrome *c* oxidase, and the rate of disappearance of the ESR signal was observed. The low rate of reduction of the spin label by ascorbate was enhanced by addition of TMPD probably because the more apolar TMPD molecule has easier access to the DCCD binding site than ascorbate. Cytochrome *c* did not affect the rate of spin-label reduction by either ascorbate or ascorbate plus TMPD despite the fact that the oxidase was inhibited with cyanide, which should ensure that the redox centers of the enzyme became rapidly reduced. This observation has implications for the structural relationship of the electron and proton-conducting pathways as discussed below.

Discussion

The observation of inhibition by DCCD of H^+ translocation via cytochrome *c* oxidase linked to specific interaction of this substance with subunit

Table III. Effects of Various Reductants on the Intensity of the Spin-Label Signal from NCCD Bound to Cytochrome *c* Oxidase[a]

Reductant	First-order rate constant of signal loss ($sec^{-1} \times 10^3$)
Ascorbate	85.5
Ascorbate + cytochrome *c*	87.7
Ascorbate + TMPD	177
Ascorbate + TMPD + cytochrome *c*	169

[a]To 0.2 ml of NCCD-labeled cytochrome *c* oxidase (4 nmol of enzyme) suspended in 1.5% sodium cholate, 50 mM sodium phosphate, 2 mM KCN, pH 7.4, was added 1 mM sodium ascorbate; 1 mM sodium ascorbate and 0.2 mM TMPD; 1 mM sodium ascorbate and 25 μM cytochrome *c* or 1 mM sodium ascorbate, 0.2 mM TMPD, and 25 μM cytochrome *c*. The change in height of the low-field peak in the ESR spectrum measured as described in the Materials and Methods section was followed with time immediately following these additions.

III of the enzyme is potentially of considerable interest in implicating this subunit in the H^+ pumping mechanism. This observation has, however, been criticized recently on two accounts. First, it has been asserted by Coin and Hinkle (1979) that inhibition of H^+ translocation by DCCD is not due to a direct interaction of this substance with the enzyme but results from the presence of small amounts of an uncoupling substance in commercially available DCCD. The DCCD used by us in the experiments reported here and elsewhere gives only one iodine-stainable spot following thin-layer chromatography in benzene:ethyl acetate (9:1 vol/vol, data not shown), indicating that it has a high degree of purity. It is conceivable, however, that minute traces of an uncoupler might not be detected by this method and at the relatively high concentrations of DCCD required to obtain inhibition, this might exert a strong effect. Direct evidence excluding this possibility is shown in Fig. 1 and Table I. There, it is clear that the strong (~75%) inhibition of cytochrome *c*-induced H^+ extrusion by DCCD is accompanied by, if anything, a decrease in the initial rate of H^+ backflux following H^+ extrusion, showing that an uncoupling phenomenon cannot account for this inhibition. Furthermore, the fact that there is no shortfall on the expected 1 H^+ consumed per ferrocytochrome *c* oxidized in either the control or the DCCD-containing samples when CCCP is present excludes the possibility that DCCD induces a net uptake of protons by the oxidase on cytochrome *c* oxidation, hence causing an artifactual appearance of inhibition of the H^+ pump.

A second point of criticism has come from our use of only relatively low stoichiometries of radio-labeled DCCD to cytochrome *c* oxidase in demonstrating its specific binding to subunit III. Capaldi and his co-workers have found that, in their hands, at higher concentrations, DCCD, while binding with most affinity to subunit III in reconstituted vesicles, also exhibits binding to other subunits, in particular subunit II (Steffens *et al.*, 1980; Prochaska *et al.*, personal communication). The experiment of Fig. 2, however, shows that at a DCCD-to-cytochrome *c* oxidase stoichiometry where an inhibition of H^+ translocation of approximately 60% is obtained (see Table II) and higher than that at which Steffens *et al.* (1980) observed inhibition of H^+ translocation, only subunit III was distinctively labeled. It should, however, be noted that, owing to the incomplete resolution of subunits II and III on the gel, it cannot be completely excluded that a minor labeling of II may have occurred. The finding of nonspecific binding by Steffens *et al.* may be due to their having too high a concentration of organic solvents in their vesicle suspensions used to dissolve DCCD during labeling. Such a situation results in subunits other than only subunit III being labeled (Casey, Broger, Thelen, and Azzi, unpublished observations).

We feel that the above evidence provides strong support for inhibition by DCCD of the cytochrome *c* oxidase proton pump through specific binding to

subunit III. An important role for subunit III in H^+ translocation is supported by the findings of Saraste *et al.* (1980) that depletion of this subunit from cytochrome *c* oxidase causes the enzyme to lose its H^+-pumping function while retaining full oxidative activity.

In addition, cytochrome *c* oxidase from *Paracoccus denitrificans* has no subunit corresponding to subunit III of the mammalian enzyme and also appears to lack an H^+-translocating activity (Ludwig, 1980).

We have proposed previously that DCCD binds to the enzyme at a apolar site, and this is confirmed by the observation that the highly polar ascorbate reduces bound NCCD, the spin-labeled analogue of DCCD, quite slowly, whereas the reduction rate is increased by addition of the more apolar TMPD (see Table III).

An interesting question concerning the H^+ pump is the proximity of the H^+-conducting pathway to that of electron transfer. To gain an insight into this point, the effect of cytochrome *c* on the rate of reduction of bound NCCD was observed (see Table III). The inability of cytochrome *c* to bring about additional spin-label reduction may indicate that the NCCD binding site does not lie directly on the pathway of electron transfer from cytochrome *c* to oxygen. This observation would be consistent with the energy released on cytochrome *c* oxidation being transmitted to the H^+-translocation apparatus via a conformational change in the protein of the kind observed by Wikström and co-workers (Wikström, 1977; Wikström and Saari, 1977).

In conclusion, the above studies establish that DCCD does indeed exert an inhibition of H^+ translocation by cytochrome *c* oxidase through binding specifically to subunit III of the enzyme and that the DCCD binding site is hydrophobic and may not lie on the electron transferring pathway.

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