

The binding of dicyclohexylcarbodiimide to cytochrome *b* of complex III isolated from yeast mitochondria

Diana S. Beattie and Liviu Clejan

Department of Biochemistry, Mount Sinai School of Medicine, City University of New York, 1 Gustave Levy Place, New York, NY 10029, USA

Received 13 October 1982

Dicyclohexylcarbodiimide binding Cytochrome b Complex III Proton translocation
Electron transport Mitochondrial respiratory chain

1. INTRODUCTION

The cytochrome *b*-*c*₁ complex (complex III) of the mitochondrial respiratory chain catalyzes electron transport, coupled to ATP synthesis and ion transport, from coenzyme Q to cytochrome *c*. This span of the respiratory chain has been shown to eject protons with an observed stoichiometry of $H^+/2e^-$ approaching 4 when studied in mitochondria in which other proton-conducting pathways of the inner membrane are selectively inhibited [1–3]. Moreover, similar stoichiometries have been observed when purified cytochrome *b*-*c*₁ complexes from either heart [4,5] or yeast [6] are reconstituted into proteoliposomes. Two different mechanistic models have been proposed to explain proton ejection in mitochondria. Mitchell's Q cycle [7,8] postulates that proton ejection occurs by a ligand conduction mechanism in which coenzyme Q is the only proton carrier. Papa, however, originally suggested that some unidentified polypeptide of the cytochrome *b*-*c*₁ complex might undergo a protonation-deprotonation cycle coupled to the oxidation-reduction reaction, such that protons were translocated across the membrane [9]. Subsequently, the cytochrome *b* dimer was proposed as the proton pump based on the observation that its redox potential displayed a pH dependence in the physiological range [10].

We have reported that dicyclohexylcarbodiimide (DCCD), the well-established carboxyl-modifying reagent, inhibited the proton-translocating device

in the yeast cytochrome *b*-*c*₁ complex reconstituted into liposomes [6]. The electrogenic ejection of protons was blocked in the DCCD-treated *b*-*c*₁ complex as well as reversed electron flow from cytochrome *b* to coenzyme Q, driven by a K^+ -diffusion potential. Similar inhibitions of proton translocation by DCCD at site 2 of the respiratory chain have also been reported in intact mitochondria isolated from both beef heart [12] and rat liver [13]. It was thus of some interest to investigate the possibility that the inhibition of proton translocation by DCCD in the *b*-*c*₁ complex involves a specific covalent linking of DCCD to the enzyme as has been shown for H^+ -translocation ATPases [14–17], cytochrome *c* oxidase [18,19] and other proton-translocating proteins [20]. The results obtained indicate that DCCD binds selectively to cytochrome *b* in complex III, suggesting that this protein is involved in proton translocation at site 2 of the respiratory chain.

2. EXPERIMENTAL

Complex III was purified from mitochondria isolated from commercial baker's yeast (Red Star), as in [21]. The preparation used in these studies contained 5.6 nmol heme *b* and 3.3 nmol heme *c*₁/mg protein and catalyzed antimycin-sensitive cytochrome *c* reduction with a specific activity of 12.6 nmol·min⁻¹·mg⁻¹ using the decyl analogue of coenzyme Q as substrate.

2.1. Binding of [14 C]DCCD to the b - c_1 complex

Aliquots (200 μ l) of complex III containing 2.2 nmol cytochrome b /ml were incubated in 100 mM KCl containing 3 mM K^+ -Hepes (pH 7.2) with [14 C]DCCD for 3 h at 12°C. The reaction was terminated by addition of 2.0 ml chloroform:methanol (1:4, v/v) and the samples immediately centrifuged for 5 min at 3000 rev./min in a bench-top centrifuge [18]. The supernatants were discarded and the pellets washed with 2.0 ml chloroform:methanol mixture and recentrifuged. The pellets were dissolved in 200 μ l 5% sodium dodecyl sulfate (SDS)-10% glycerol in 50 mM Tris-HCl (pH 6.8). Aliquots of 40 ml were added to 10 ml Liquiscint (National Diagnostics) for determination of radioactivity.

2.2. Determination of the [14 C]DCCD-binding subunit(s)

Complex III (containing 2.4 nmol of cytochrome b) was incubated in 1.0 ml of 100 mM KCl, 3 mM K^+ -Hepes (pH 7.2) with 240 nmol [14 C]DCCD for 3 h at 10–12°C. The incubation mixture was layered onto 4.0 ml 10% sucrose and then centrifuged for 3 h at 140000 $\times g$. The resulting red pellet was dissolved in 100 μ l 5% SDS, 10% glycerol, 50 mM Tris-HCl (pH 6.8) containing 5% mercaptoethanol, and allowed to stand overnight prior to electrophoresis on 11%, 12.5% and 15% polyacrylamide gels, as in [21]. The gels were stained with Coomassie blue and destained and then scanned at 590 nm in an EC gel scanner. The gels were then frozen and sliced into 1 mm slices. The slices were incubated for 5 h at 65°C with 0.3 ml 30% H_2O_2 and radioactivity determined in 10 ml Liquiscint.

2.3. Reagents

[14 C]DCCD (54 mCi/mmol) was obtained from the Centre d'Etudes Nucléaires de Saclay (Gif-sur-Yvette) and stored in methanol at -25°C. All other reagents were of the highest quality commercially available.

3. RESULTS AND DISCUSSION

[14 C]DCCD was bound to the isolated complex III in a concentration-dependent manner (fig. 1). The incubation conditions used (medium, temperature and time) were identical to those used

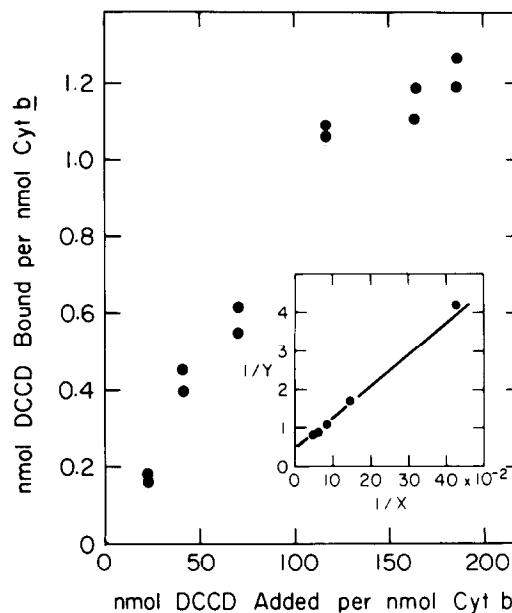


Fig. 1. Binding of [14 C]DCCD to the b - c_1 complex: 200 μ l aliquots of complex III containing 0.44 nmol cytochrome b were incubated with increasing amounts of [14 C]DCCD for 1 h at 12°C. The reaction was stopped by addition of 2.0 ml chloroform:methanol (1:4, v/v) and the samples prepared for radioactive counting. The inset presents the results obtained from the above, averaged data as a double reciprocal plot.

previously to demonstrate the inhibition of proton translocation by DCCD [6]. Plotting the data in a double reciprocal plot resulted in a straight line with an intercept on the y -axis which indicated a maximum binding of 2.08 nmol DCCD/mol cytochrome b .

The binding of DCCD to other proton translocating enzymes has been shown to be covalent. Washing with chloroform:methanol did not remove the radiolabeled DCCD, suggesting that DCCD is also bound covalently to complex III (fig. 1). To determine to which subunit the [14 C]DCCD was found, the complex was separated into its component subunits by SDS-polyacrylamide gel electrophoresis after the incubation with radioactive DCCD. The washing procedure after the incubation was modified so that the complex was centrifuged through 10% sucrose to remove unbound DCCD prior to solubilization in dissociation buffer. In addition, to minimize non-specific

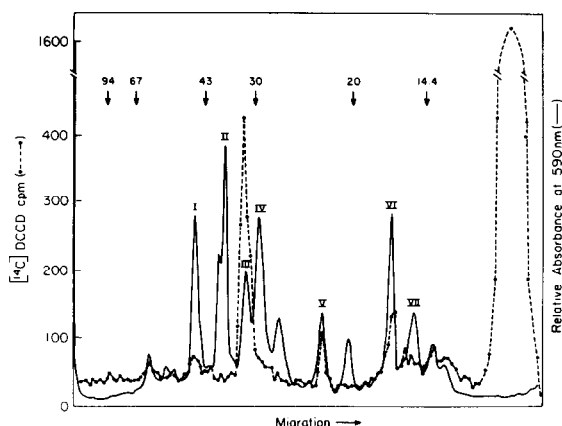


Fig. 2. Identification of the [^{14}C]DCCD binding subunit(s) by SDS gel electrophoresis. Complex III was incubated with 100 nmol [^{14}C]DCCD/nmol cytochrome *b* for 3 h at 10–12°C. The pellets obtained after centrifugation through 10% sucrose were suspended in 100 μl dissociation buffer. A 25 μl aliquot was analyzed on a 15% acrylamide gel: (○—○) densitometric pattern of the Coomassie Blue stained bands; (○---○) radioactive counts of 1 mm slices obtained from the same gel; the Roman numerals represent the subunits of complex III [21]; (→) $M_r \times 10^{-3}$.

binding of DCCD, a less than maximal concentration of DCCD (100 nmol/nmol cytochrome *b* was chosen. Fig. 2 indicates that only subunit III of the cytochrome *b*-*c*₁ complex contained significant amounts of [^{14}C]DCCD while subunits V and VI were slightly labeled. The large radioactive peak migrating near the front appears to be free [^{14}C]DCCD as no subunit of the *b*-*c*₁ complex migrates in that region of the gel. Furthermore, free [^{14}C]DCCD also displays a similar migration pattern (not shown).

Subunit III of the *b*-*c*₁ complex from yeast mitochondria has been identified as cytochrome *b* on the basis of its mobility in gels of different acrylamide concentrations and its isoelectric point [21]. To confirm that the subunit labeled with [^{14}C]DCCD observed on the 15% acrylamide gel in fig. 2 is cytochrome *b*, the same labeled complex was analyzed on 11% and 12.5% acrylamide gels, so that the app. M_r -values of subunits III and IV could be determined. Subunit III clearly demonstrates the anomalous migrating pattern previously reported for cytochrome *b* [21–24] (table 1). In

Table 1

Apparent M_r -values of subunits III and IV of complex III

Acrylamide	Subunit III	Subunit IV
11%	30 000	30 000
12.5%	31 500	29 500
15%	34 000	30 000

SDS-dissociated complex III (50 μg) was analyzed on 11%, 12.5% and 15% polyacrylamide gels and the M_r -values of the subunits determined, as in [24]

11% acrylamide gels, subunits III and IV could not be resolved while, at increasing acrylamide concentrations, subunit III migrated progressively more slowly with an increasing app. M_r . We conclude that cytochrome *b* is the subunit of the complex III to which [^{14}C]DCCD is covalently bound.

The DCCD inhibition of proton ejection in the *b*-*c*₁ complex [6], plus the covalent linkage of DCCD to a single subunit of the complex, suggests that the mechanism of proton translocation in this span of the respiratory chain may be similar to that of cytochrome *c* oxidase in which DCCD has also been reported to inhibit proton ejection [18,19]. DCCD has been shown to bind to a single glutamate residue present in subunit III of cytochrome *c* oxidase [19]. The sequence of amino acids surrounding this glutamate residue is strikingly similar to the sequences reported for the DCCD-binding site in the proteolipid protein of both yeast and beef heart mitochondrial ATP synthetases [25]. These results make it tempting to speculate that DCCD may block proton movement in the respiratory chain complexes by a similar mechanism to that proposed for H^+ -translocating ATPases [26]. The binding of [^{14}C]DCCD exclusively to cytochrome *b* in complex III, reported here, plus the DCCD inhibition of electrogenic proton ejection in complex III reconstituted into liposomes [6], suggest a major role for cytochrome *b* in proton translocation at this site in the respiratory chain.

ACKNOWLEDGEMENTS

This study was supported, in part, by National Institutes of Health Grant HD-04007 and was

started while D.S.B. was a Fogarty International Fellow (FO6-TW 00616) in the Laboratory of Dr André Goffeau at the Université de Louvain, Belgium. We express our thanks to Dr Anita Sidhu for help in the preparation of the *b*-*c*₁ complex and to Dr André Goffeau for his continued interest.

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