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

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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 $H^+/2\ e^-$ 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 $12.6\ nmol \cdot min^{-1} \cdot mg^{-1}$ using the decyl analogue of coenzyme Q as substrate.

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2.1. *Binding of [^{14}C]DCCD to the* b–c_1 *complex*

Aliquots (200 μl) 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 μl 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 × *g*. The resulting red pellet was dissolved in 100 μl 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 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*.

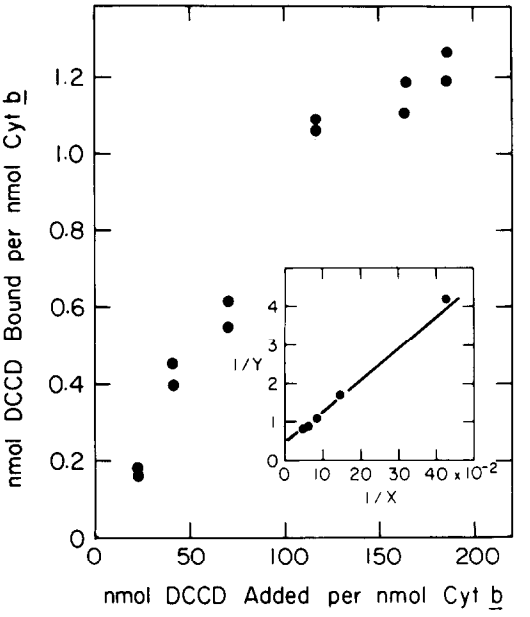

Fig. 1. Binding of [^{14}]DCCD to the *b*–c_1 complex: 200 μl aliquots of complex III containing 0.44 nmol cytochrome *b* were incubated with increasing amounts of [^{14}C]DCCD for 1 h at 12°C. The reaction was stopped by addition of 2.0 ml chloroform : methanol (1 : 4, v/v) and the samples prepared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

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

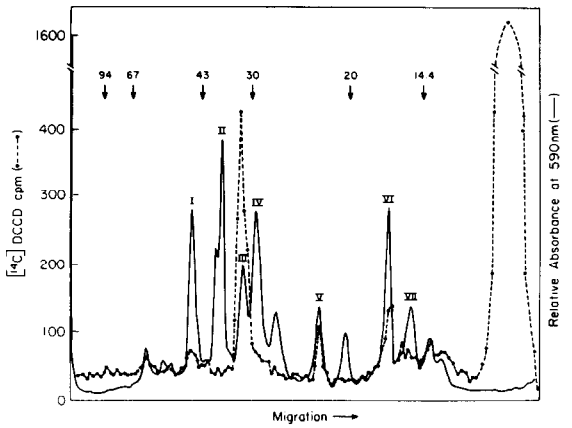

Fig. 2. Identification of the [^{14}C]DCCD binding subunit(s) by SDS gel electrophoresis. Complex III was incubated with 100 nmol [^{14}]DCCD/nmol cytochrome *b* for 3 h at 10–12°C. The pellets obtained after centrifugation through 10% sucrose were suspended in 100 μl dissociation buffer. A 25 μl aliquot 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%	30000	30000
12.5%	31500	29500
15%	34000	30000

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.

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