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Further Studies on the Binding of DCCD to Cytochrome *B* and Subunit VIII of Complex III Isolated from Beef Heart Mitochondria

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Abstract

Complex III (the cytochrome b–c_1 complex) from beef heart mitochondria was incubated with [^{14}C]DCCD for various periods of time. The polypeptide profile of the complex was compared in both stained gels and their autoradiograms when three different methods were used to terminate the reaction. Precipitation with ammonium sulfate resulted in the formation of a new band with an apparent molecular weight of 39,000 in both incubated samples and the zero time controls. Reisolation of the complex by centrifugation through 10% sucrose or by precipitation with trichloroacetic acid did not result in any changes in the appearance of the subunit peptides of the complex. Subunit III (cytochrome b) and subunit VIII were the only bands labeled after termination of the reaction by centrifugation through sucrose, while both ammonium sulfate and trichloroacetic precipitation resulted in nonspecific labeling of several other subunits of the complex and increased labeling of subunit VIII relative to subunit III. Preincubation of the complex with antimycin prior to treatment with [^{14}C]DCCD resulted in a 50% decrease in the binding of DCCD to both cytochrome b and subunit VIII. Furthermore, treatment of the complex III with DCCD resulted in a change in the red shift observed after antimycin or myxothiazol addition to the dithionite-reduced complex resulting in a broad peak with no sharp maximum. These results provide further confirmation that DCCD binds preferentially to cytochrome b and subunit VIII of complex III from beef heart mitochondria and suggest that cytochrome b may play a role in proton translocation.

Key Words: Mitochondria; cytochrome; Complex III; respiratory chain; proton pumping.

Introduction

In recent years, dicyclohexylcarbodiimide (DCCD), the well-known carboxyl modifying reagent, has been widely used to study the mechanism of proton

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translocation in a variety of enzyme complexes. DCCD was initially reported to inhibit the movement of protons in the F_0–F_1 ATPases by covalent binding to a specific glutamyl or aspartyl residue (Fillingame, 1980). Subsequently, DCCD was reported to block electrogenic proton translocation in both cytochrome *c* oxidase (Casey *et al.*, 1980; Prochaska *et al.*, 1981) and the cytochrome b–c_1 region (Beattie and Villalobo, 1982; Esposti *et al.*, 1982; Price and Brand, 1983) of the respiratory chain. In the latter, site 2 of the chain, it was demonstrated that DCCD inhibits the proton translocating device without significantly affecting the rate of electron transfer in either the purified complex reconstituted into liposomes (Clejan and Beattie, 1983) or in intact mitochondria (Esposti *et al.*, 1983; Lorusso *et al.*, 1983; Clejan *et al.*, 1984a, b), suggesting that these two processes can be uncoupled.

These specific inhibitory effects of DCCD on proton translocation in the cytochrome b–c_1 region of the respiratory chain have prompted investigations of the possibility that a covalent linking of DCCD to one or more subunits of complex III may occur, as has been demonstrated with other proton translocating enzyme complexes (Senior, 1983). Initially, it was shown in this laboratory that radioactive DCCD binds selectively to cytochrome *b* in yeast complex III, suggesting that this protein is involved in proton translocation at this site of the respiratory chain (Beattie and Clejan, 1982; Beattie *et al.*, 1984). Contrasting results were reported, however, with complex III isolated from beef heart mitochondria. Initially, the binding of radioactive DCCD to all subunits of this complex was reported with a preferential binding to cytochrome *b* in the reconstituted complex (Nalecz *et al.*, 1983). Under certain conditions, cross-linking was observed between subunits V (the iron–sulfur protein) and VII after treatment with DCCD. Subsequently, Lorusso *et al.* (1983) reported that DCCD was bound preferentially to the 8-kDa subunit of the complex concomitant with cross-linking of both the 8- and 12-kDa subunits to the iron–sulfur protein. By contrast, Esposti *et al.* (1983) reported that the preferential binding of DCCD to the 8-kDa peptide (subunit VIII) correlated with the selective inhibition of electrogenic proton ejection. Recently, we reported that DCCD binds to both cytochrome *b* and subunit VIII of the beef heart complex III in a time- and concentration-dependent manner. No cross-linking was observed after DCCD treatment unless the complex was reisolated by precipitation with ammonium sulfate. In the present report, various conditions for stopping the reaction of the beef heart mitochondrial complex III with DCCD have been compared. In addition, the effects of antimycin on the binding of DCCD plus the spectral shifts of cytochrome *b* were investigated.

Experimental

Isolation of Complex III

Complex III, isolated from beef heart mitochondria by the procedure of Rieske (1967), containing 5.1 nmol of heme *b* and 4.0 nmol of heme c_1/mg protein was a generous gift from Dr. Bernard Trumpower. Complex III from yeast mitochondria was prepared by the method of Sidhu and Beattie (1982) and contained 2.8 nmol of heme *b* and 1.2 nmol of heme c_1/mg protein.

Treatment with DCCD

Complex III from beef heart mitochondria (100–200 γ) was incubated with [^{14}C]DCCD in a medium containing 100 mM KCl, 3 mM K^+-Hepes, pH 7.2, for 1 h at 12°C. The incubation was terminated by three different methods: (1) by a 1 : 10 dilution with ice-cold 10% sucrose followed by centrifugation at 106,000 × *g* for 2.5 h, (2) by the addition of 10% trichloroacetic acid followed by centrifugation for 5 min in a microfuge and the pellets thus obtained washed twice with water, or (3) by the addition of 50% saturated ammonium sulfate followed by centrifugation for 5 min in a microfuge. The pellets obtained by all three methods were dissociated in 5% SDS, 50 mM Tris-Cl, pH 6.8, 10% glycerol, 2 mM EDTA, and 5% β-mercaptoethanol overnight at room temperature prior to SDS gel electrophoresis, autoradiography, and salicylate enhancement (Beattie *et al.*, 1984). An E and C densitometer with an attached Hewlett-Packard 3390 A integrator was used to scan the Coomassie-Blue stained gels and the autoradiograms. The relative radioactivity and protein content were obtained from the computer-calculated areas below the curve. Specific activities represent the relative radioactivity divided by the relative protein content. It should be noted that these are all relative values and depend to some extent on the length of time that the gel was exposed to the film during the autoradiography.

Complex III was treated with 10 nmol of antimycin per nmol of cytochrome *b* for 10 min at 12°C. The control was treated with an equivalent amount of methanol. Subsequently 50 nmol of [^{14}C]DCCD/nmol of heme *b* was added to both samples and the incubation continued for 1 h at 12°C.

Spectra

Spectral analyses were performed at room temperature with a Hitachi double-beam, double-wavelength model 557 spectrophotometer as described previously (Clejan *et al.*, 1983). Complex III containing a total of 0.1 nmol of cytochrome *b* was incubated with 10 nmol of DCCD for 1 hr at 15°C prior to reduction with dithionite as described in the figure legend.

Materials

Aliquots of [^{14}C]DCCD obtained from Amersham (62 mCi/mmol) in toluene were evaporated to dryness under a stream of nitrogen and redissolved in methanol just prior to the start of the experiment. Acrylamide, twice recrystallized, was obtained from Serva. Kodak X-ray XAR-5 film was used for autoradiography and venturicidin was from Gallard Schlesinger. Myxothiazol was a generous gift from Dr. Wolfram Trowitzsch.

Results

In previous studies (Beattie *et al.*, 1984; Clejan *et al.*, 1984a), we reported that treatment of complex III isolated from either beef heart or yeast mitochondria with DCCD did not result in any changes in the appearance of the various subunits when the complex was reisolated after the incubation by centrifugation through 10% sucrose. Under these mild conditions, no new bands were observed, suggesting that cross-linking of different subunits had not occurred. By contrast, stopping the incubation of the beef heart complex with DCCD by precipitation with ammonium sulfate resulted in the formation of a novel band even in the zero time control (Clejan *et al.*, 1984a). In the current study, we decided to reinvestigate the effects of three different methods commonly used to terminate the incubation of beef heart complex III with DCCD on both the protein profile of the complex and on the labeling pattern of the different subunits with the inhibitor. In these experiments, only one incubation with DCCD was performed from which aliquots were removed at appropriate intervals. The reaction was stopped by precipitation with either trichloroacetic acid or ammonium sulfate or alternately diluted in 10% sucrose prior to centrifugation for 2.5 hr as described under Methods. As seen in Fig. 1, precipitation of the beef heart complex III with trichloroacetic acid or ammonium sulfate in the absence of DCCD had no effect on the appearance of the complex after SDS-gel electrophoresis as compared to the untreated complex (Fig. 1, lanes 3 and 4 compared to lane 2). By contrast, a novel polypeptide with a molecular weight of approximately 39,000 was observed when complex III was treated with DCCD and precipitated with ammonium sulfate (Fig. 1, lanes 7 and 10). The novel band appeared with equal intensity in both the zero-time controls and the samples after a 30-min incubation with DCCD. A slight change in both the mobility and the staining intensity of subunit V (the iron–sulfur protein) was also observed in the samples precipitated with ammonium sulfate. No differences in the polypeptide profile were observed when the complex was reisolated by precipitation with trichloroacetic acid or by centrifugation through sucrose after the incubation with DCCD.

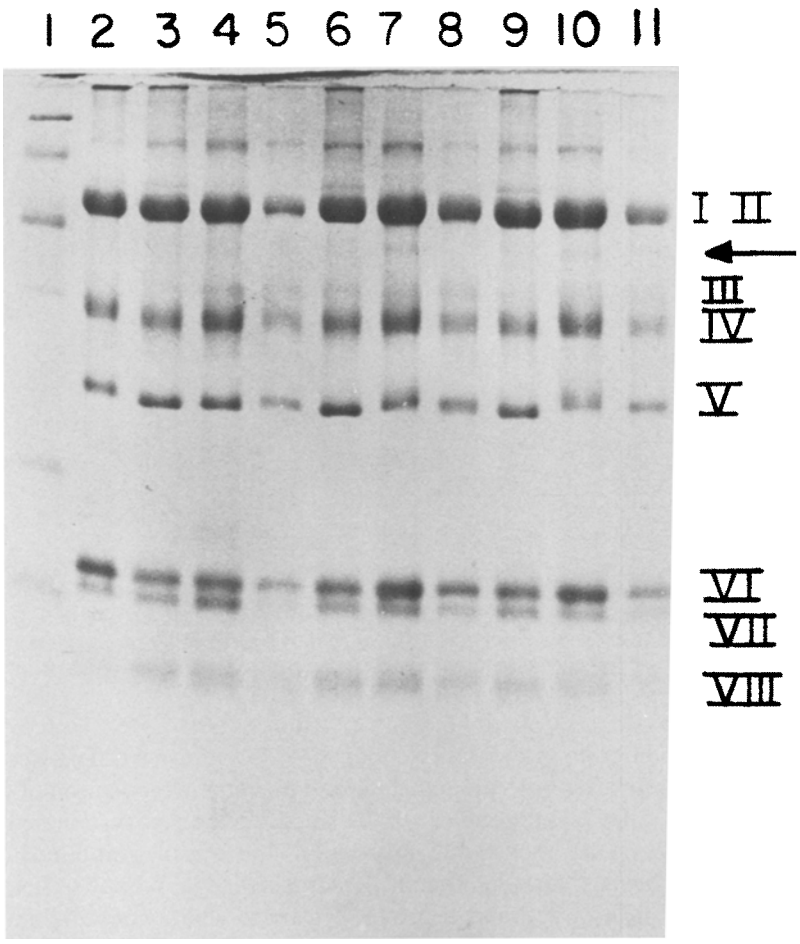


Fig. 1. Lack of cross-linking of the subunits of complex III after DCCD treatment. Beef heart mitochondrial complex III was incubated in 100 mM KCl, 3 mM K^+-Hepes, pH 7.2, with 50 nmol [^{14}C]DCCD/nmol of cytochrome *b*. Lane 1, molecular weight standards (94,000, 67,000, 43,000, 30,000, 20,000, and 14,000). Lane 2, untreated complex III. Lanes 3, 4, and 5 contain complex III precipitated with trichloroacetic acid, precipitated with ammonium sulfate, or centrifuged through sucrose, respectively. Lanes 6, 7, and 8 contain complex III incubated with [^{14}C]DCCD for 0 min followed by precipitation with trichloroacetic acid, precipitation with ammonium sulfate, or centrifugation through sucrose, respectively. Lanes 9, 10, and 11 contain complex III incubated with [^{14}C]DCCD for 30 min followed by precipitation with trichloroacetic acid, precipitation with ammonium sulfate, or centrifugation through sucrose, respectively. The arrow in the figure indicates the novel peptide observed.

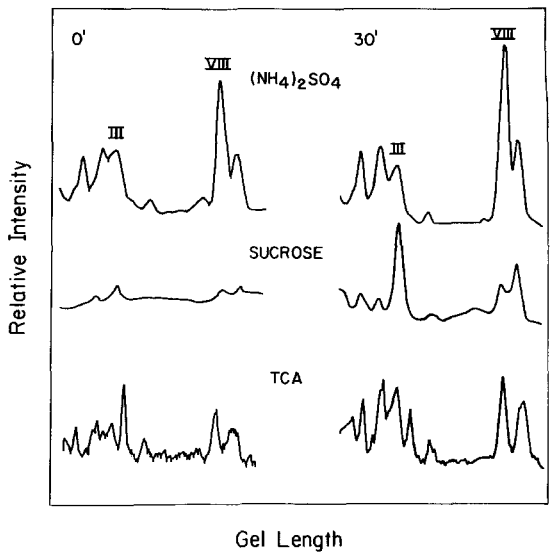


Fig. 2. Nonspecific labeling of the subunits of beef heart complex III after treatment with DCCD. Beef heart mitochondrial complex III was incubated with [^{14}C]DCCD at 0 time or for 30 min as described in the legend to Fig. 1. The reaction was stopped by precipitation with ammonium sulfate, by centrifugation through sucrose, or by precipitation with trichloroacetic acid as indicated.

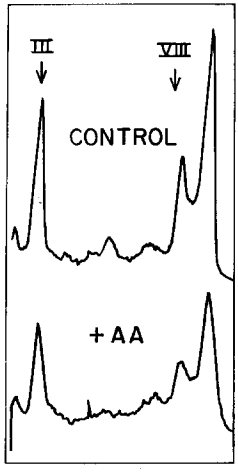
The autoradiogram of the same gel of Fig. 1 also revealed differences in the labeling pattern after both the zero-time and the 30-min incubation of the complex with [^{14}C]DCCD depending on the method chosen to terminate the reaction (Fig. 2). After the 30-min incubation, the complex obtained by centrifugation through sucrose contained radioactivity in subunits III and VIII as well as in a large band near the dye front which contains phospholipids (Beattie *et al.*, 1984). Previously, we had established that DCCD labeled these two bands in complex III islolated from beef heart mitochondria in both a time- and concentration-dependent manner (Clejan *et al.*, 1984a). Similar results were obtained in the present study (Fig. 2) in the samples reisolated by centrifugation through sucrose. By contrast, stopping the reaction by precipitation with either ammonium sulfate or trichloroacetic acid resulted in nonspecific labeling of other subunits of the complex including the core proteins. In addition, subunit VIII was labeled with [^{14}C]DCCD to a much greater extent (specific activity 3.94 after a 30-min incubation) when ammonium sulfate was used to precipitate the complex as compared to a specific activity of 1.07 when the complex was reisolated by centrifugation through sucrose. An identical amplification was used to scan each lane of gel, and hence they can be directly compared. Furthermore, an almost identical

pattern of labeling and similar specific activities of both subunits III and VIII were observed in the zero-time controls obtained by precipitation with ammonium sulfate, suggesting that precipitation of the complex under these conditions allows nonspecific reactions to occur with DCCD even at 4°C (Fig. 2). Similarly, several bands were labeled in the zero-time controls precipitated with trichloroacetic acid. It should be noted that no significant labeling of subunits III and VIII or the band corresponding to phospholipids occurred in the zero-time control when the reaction was stopped by centrifugation through sucrose, indicating that dilution of the complex with the sucrose effectively prevents any further reactions with the DCCD. Similar results were also obtained when the complex was reisolated by centrifugation through 1 ml Sephadex G-50 columns (Hotscher and Capaldi, 1984) as first described by Penefsky (1977). These results further confirm our previous suggestion that the method used to reisolate complex III after incubation with [^{14}C]DCCD is critical and may explain some of the differences reported by other workers in the field.

Effect of Antimycin on the Binding of DCCD to Complex III

In a previous study, we reported that preincubation of complex III from yeast mitochondria with antimycin blocked by 90% the subsequent binding of [^{14}C]DCCD to cytochrome *b* (Beattie *et al.*, 1984). In a similar experiment in which complex III from beef heart mitochondria was preincubated for 10 min with antimycin prior to addition of [^{14}C]DCCD and incubated for 30 min, an approximately 50% decrease in the labeling of both subunits III and VIII was observed (Fig. 3). Similar decreases in the binding of DCCD were observed after 15 or 60 min of incubation with DCCD after the initial preincubation with antimycin.

The reverse effect of DCCD on the binding of antimycin to cytochrome *b* was also examined. Previously, we had reported that DCCD treatment of either isolated complex III, yeast mitochondria (Clejan and Beattie, 1983), or rat liver mitochondria (Clejan *et al.*, 1984b) resulted in a broadening in the peak of the red shift observed after antimycin addition, suggesting that DCCD caused a modification of cytochrome *b* which altered the subsequent binding of antimycin. As seen in Fig. 4 (trace 2), reduction of complex III obtained from beef heart mitochondria with dithionite resulted in a similar sharp peak at 561.5 nm due to cytochrome *b* in both the control and DCCD-treated complexes. Trace 4 was obtained after the addition of antimycin with the dithionite-reduced complex as the baseline (trace 3). In the control, a sharp peak at 565 nm was observed with a trough at 569 nm. In the DCCD-treated complex, addition of antimycin resulted in an initial shift to 565.5 nm in the first 30 sec; however, within less than 2 min the peak had broadened with an apparent maximum at 575 nm.



Sp. Act		
	CONTROL	AA
III	4.61	2.60
VIII	8.62	3.03

Fig. 3. Effect of antimycin on the binding of DCCD to complex III. Beef heart mitochondrial complex III was incubated with 10 nmol of antimycin/nmol of cytochrome *b* for 10 min at 12°C in the incubation medium described in the legend to Fig. 1. Subsequently, 50 nmol of DCCD/nmol of cytochrome *b* was added, the incubation continued for 30 min at 12°C, and stopped by centrifugation through sucrose. The Coomassie-blue stained gel and the corresponding autoradiogram were scanned and the specific activities calculated as described in the text. The higher specific activities calculated in this gel compared to that of Fig. 2 resulted from a longer exposure of the autoradiogram.

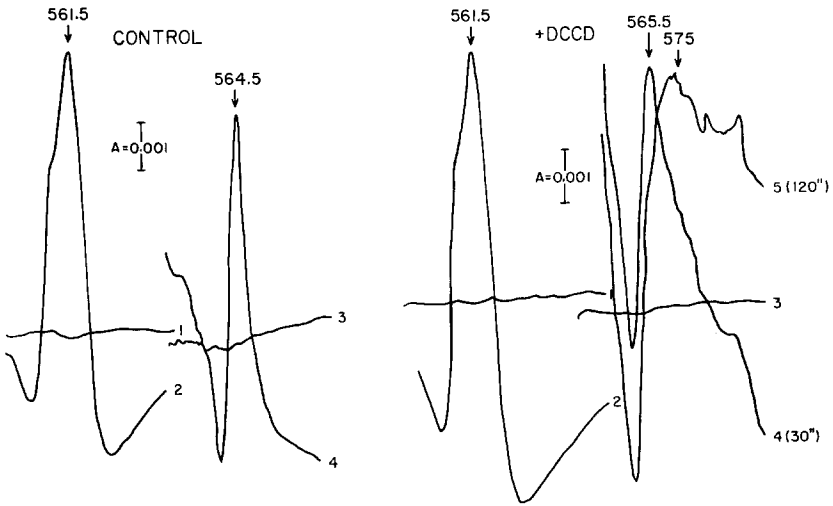


Fig. 4. Effects of DCCD on the antimycin-induced red shift. Beef heart mitochondrial complex III was incubated in 100 mM KCl, 3 mM K-Hepes, pH, 7.2, with 50 nmol DCCD/nmol cytochrome *b* for 1 h at 12°C. The complex was diluted with 0.1 M sodium phosphate, pH 7.2, to give 4.7 μM cytochrome *b*. A slit width of 1 nm with a 2-times expanded wavelength scale was used. Control (left): trace 1, baseline; trace 2, dithionite-reduced versus air-oxidized; trace 3, baseline; trace 4, 4 μM antimycin was added. DCCD-treated (right): the same as the control except that trace 4 was recorded 30 sec after the addition of antimycin, and trace 5 after 2 min.

The effects of DCCD treatment of complex III on the spectral shifts induced by myxothiazol were also investigated (Fig. 5). To obtain the spectral shifts with myxothiazol previously described (Becker *et al.*, 1981; von Jagow and Engel, 1981), it was necessary to resuspend the complex in a solution containing Triton X-100 (von Jagow *et al.*, 1984). A sharp peak with an absorption maximum at 568 nm was observed when myxothiazol was added to the dithionite-reduced complex (Fig. 5, trace 4 of the control). Addition of myxothiazol to the DCCD-treated complex resulted in the appearance of a broad band with an apparent maximum at 572 nm. The sharp trough observed at 559 nm in the control was also broadened and less extensive in the DCCD-treated complex. The addition of the hydrophobic DCCD did not cause any noticeable turbidity in the cuvette nor did it change the absorption spectrum of the complex. The only changes noticed were in the response to the addition of the two inhibitors.

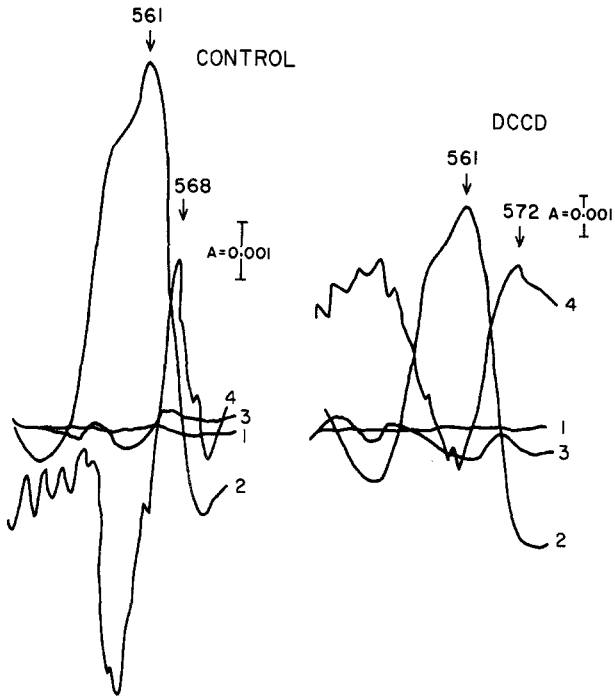


Fig. 5. Effect of DCCD on the myxothiazol-induced shift. Beef heart mitochondrial complex III was incubated with DCCD as described in the legend to Fig. 4. Before spectral assay the complex was diluted with 0.1 M sodium phosphate, pH 7.2, containing 0.5% Triton X-100. A slit width of 1 nm and a 3-times expanded wavelength scale was used. Control: trace 1, baseline; trace 2, dithionite-reduced versus air-oxidized; trace 3, baseline; trace 4; 8 μM myxothiazol was added. DCCD-treated: the traces are the same as the control.

Discussion

The results of the current study provide further evidence for the involvement of cytochrome *b* in the binding of DCCD, and hence proton translocation. We have confirmed our previous observations that DCCD binds to both cytochrome *b* and subunit VIII of complex III from beef heart mitochondria in a time- and concentration-dependent manner. Furthermore, the present data reinforce our earlier suggestion that the method used to reisolate the complex after the incubation with [^{14}C]DCCD is critical to demonstrate the binding of DCCD to these specific subunits. Precipitation with ammonium sulfate or trichloroacetic acid resulted in the nonspecific labeling of several subunits including the core proteins in addition to leading to a large increase in the specific labeling of subunit VIII. The nonspecific nature of the labeling of the other subunits was indicated by the observation that they were labeled as extensively in the zero-time control as in the 30- or 60-min incubation.

DCCD treatment also resulted in changes in the conformation of cytochrome *b*, as indicated by changes in the spectral shifts observed upon addition of either antimycin or myxothiazol to the DCCD-treated complex. Antimycin addition to the DCCD-treated complex reduced with dithionite resulted in the appearance of a broad peak with no clear maximum after 2 min as compared to the sharp stable peak observed in the control (Fig. 4). Similarly, the spectral shift observed after addition of myxothiazol to the dithionite-reduced complex was also modified in the DCCD-treated complex (Fig. 5). Previously, Becker *et al.* (1981) concluded that the red shift observed after myxothiazol treatment was due to changes in cytochrome *b*-566 and not *b*-562. In subsequent studies on the effects of myxothiazol on the oxidation–reduction reactions, spectral and thermodynamic properties of cytochromes *b*, c_1, and the iron–sulfur protein, von Jagow *et al.* (1984) concluded that myxothiazol binds to cytochrome *b*. The observation that DCCD treatment of the complex obscured the sharp spectral shift observed after either antimycin or myxothiazol addition suggests that the covalent binding of DCCD to cytochrome *b* affects this protein in such a way that both antimycin and myxothiazol cannot bind in their usual manner. It is tempting to speculate that DCCD induces, or prevents, conformational changes in cytochrome *b* which are necessary for proton ejection and also prevents the proper binding of the two inhibitors.

Acknowledgments

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