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Evidence for a Conformational Change in Subunit III of Bovine Heart Mitochondrial Cytochrome *c* Oxidase[1]

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The role of subunit III in the function of mitochondrial cytochrome *c* oxidase is not clearly understood. Previous work has shown that chemical modification of subunit III with *N,N′*-dicyclohexylcarbodiimide (DCCD) reduced the proton-pumping efficiency of the enzyme by an unknown mechanism. In the current work, we have employed biochemical approaches to determine if a conformational change is occurring within subunit III after DCCD modification. Control and DCCD modified beef heart enzyme were subjected to limited proteolysis in nondenaturing detergent solution. Subunit III in DCCD treated enzyme was more susceptible to chymotrypsin digestion than subunit III in the control enzyme. We also labeled control and DCCD-modified enzyme with iodoacetyl–biotin, a sulfhydryl reagent, and found that subunit III of the DCCD-modified enzyme was more reactive when compared to subunit III of the control enzyme, indicating an increase in reactivity of subunit III upon DCCD binding. The cross linking of subunit III of the enzyme induced by the heterobifunctional reagent, *N*-succinimidyl(4-azidophenyl -1,3′-dithio)-propionate (SADP), was inhibited by DCCD modification, suggesting that DCCD binding prevents the intersubunit cross linking of subunit III. Our results suggest that DCCD modification of subunit III causes a conformational change, which most likely disrupts critical hydrogen bonds within the subunit and also those at the interface between subunits III and I in the enzyme. The conformational change induced in subunit III by covalent DCCD binding is the most likely mechanism for the previously observed inhibition of proton-pumping activity.

KEY WORDS: Cytochrome *c* oxidase; beef heart mitochondria; *N, N′*-dicyclohexylcarbodiimide; subunit III; limited proteolysis; chemical cross linking; chemical modification of membrane proteins.

INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1), a two-heme *a*, three-copper metalloenzyme, oxidizes ferrocytochrome *c* and reduces molecular oxygen into water in the mitochondrial respiratory chain (Saraste, 1999; Babcock and Wikstrom,1992). The enzyme transduces the energy released in its redox reactions into an electrochemical gradient across the mitochondrial inner membrane, which subsequently drives the formation of ATP by the ATP synthase (Mitchell, 1979). Cytochrome *c* oxidase (COX[3]) establishes an electrochemical gradient by pumping protons vectorially across the mitochondrial membrane (Wikstrom and Krab, 1979).

[1] Key to abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; COX, cytochrome *c* oxidase; DCCD, *N, N′*-dicyclohexylcarbodiimide; DMSO, dimethylsulfoxide; HRP, horseradish peroxidase; IgG, immunoglobulin; kDa, kilo dalton; NBT, nitroblue tetrazolium; SADP, *N*-succinimidyl-(4-azidophenyl-1,3′-dithio)-propionate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline: 20 mM Tris, pH 7.5, 500 mM NaCl; TBST, Tris-buffered saline, Tween-20: 20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween-20; TMB, 3,3′,5,5′-tetramethylbenzidine; TX-100, Triton X-100.

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The mammalian mitochondrial form of COX consists of thirteen subunits. The three largest subunits are encoded by mitochondrial DNA with the ten additional subunits being encoded by nuclear DNA (Capaldi, 1990). Bacterial forms of COX contain three subunits, all strong homologs of the mitochondrially encoded subunits (Saraste, 1990). The three-dimensional structures of both the mitochondrial (Tsukihara *et al.*, 1996; Yoshikawa *et al.*, 1998) and the bacterial (Iwata *et al.*, 1995) enzymes are also known. Both heme *a* moieties, one copper atom, and the oxygen binding site are located in the largest subunit (subunit I), while a diatomic copper center and the cytochrome *c* binding site are located in subunit II (Tsukihara *et al.*, 1996). The two largest mitochondrial subunits contain the oxidation–reduction centers of the enzyme.

Although the remaining mitochondrial encoded subunit, subunit III, is conserved with high amino acid homology in most bacterial species (Saraste, 1990), the functional role of this subunit is unknown (Bratton *et al.*, 1999; Hoffbuhr *et al.*, 2000). The initial proposed function of this subunit was to facilitate the vectorial movement of protons from their site of catalytic release to the external milieu (Thompson and Ferguson-Miller, 1983; Prochaska and Fink, 1987). In analogy to a proton conduit, subunit III contains conserved anionic amino acid residues that could act as mediators to shuttle protons through the enzyme (Anderson *et al.*, 1982). The experimental data which supported this hypothesis were that dicyclohexylcarbodiimide (DCCD) bound to subunit III specifically and blocked the proton-pumping activity of the enzyme (Casey *et al.*, 1980; Prochaska *et al.*, 1981). It was later found that the amount of DCCD bound to COX was stoichiometric with inhibition of proton-pumping activity and that DCCD was bound to a specific, highly conserved membrane intercalated glutamate (Glu90) (Prochaska *et al.*, 1981). Subsequent mutagenesis of Glu90 in subunit III of *Paracoccus denitrificans* COX yielded a mutant enzyme with wild-type proton translocation activity (Haltia *et al.*, 1991). This result suggested that the mechanism of DCCD-induced inhibition of proton pumping in COX was indirect and not due to a specific blockage of a participatory amino acid residue in the proton channel of the enzyme (Musser *et al.*, 1993).

The molecular mechanism of how DCCD blocks proton pumping in COX is unclear, although recent work has suggested that subunit III is required for the stabilization of the enzyme through interactions with other subunits (Haltia *et al.*, 1994; Bratton *et al.*, 1999; Hoffbuhr *et al.*, 2000). This paper details how the covalent modification of subunit III of bovine heart COX with DCCD affects the structure of the enzyme. Our results show that DCCD induces a conformational change in subunit III and that insertion of bulky modification groups such as DCCD may perturb critical amino acid interactions at the subunit I–III interface, resulting in a loss of proton-pumping activity. Our results also lend support to the premise that structural integrity of subunit III is essential for optimal activity of the enzyme (Haltia *et al.*, 1994; Bratton *et al.*, 1999).

EXPERIMENTAL MATERIALS AND METHODS

Preparation of Cytochrome *c* Oxidase (COX)

COX was isolated from bovine heart mitochondria as described by Yonetani (1967). COX concentration (heme aa_3) was determined using an extinction coefficient of 33 mM^{-1} for reduced heme aa_3 at $\Delta A_{605-630\ nm}$ (Briggs and Capaldi, 1977). Protein concentration was estimated using the method of Lowry *et al.* (1951). The purity of the enzyme was assessed by SDS–PAGE and electron transfer activity. All preparations exhibited a nmol heme *a*/mg protein ratio of 7 to 9.5.

Gel Filtration Chromatography

COX (5.0–7.5 mg) was preincubated with 5 mg TX-100 per mg COX for 30 min at 0°C and was then chromatographed over a Sepharose 2B column (1.75 × 30 cm) equilibrated with 20 mM $KHPO_4$, pH 7.2, 0.1% TX-100, 90 mM KCl. Fractions with absorbance at 420 nm were pooled and the heme aa_3 concentration determined.

Reaction of COX with DCCD

After gel filtration, COX (1 μM aa_3) was reacted at room temperature for 1 h with increasing concentrations (0–0.62 mM) of DCCD (dissolved in methanol) (Aldrich Chemical) in 20 mM $KHPO_4$, pH 7.2, 90 mM KCl, 0.1% TX-100. Reactions were quenched with 100 mM succinate at 0°C for 15 min.

Limited Proteolysis of Control and DCCD-Treated COX with Chymotrypsin

After preincubation in TX-100, COX was diluted with 20 mM $KHPO_4$, pH 7.2, 90 mM KCl, 0.1% TX-100 to a final concentration of 3.4 to 5.0 μM heme aa_3. The diluted enzyme solution was then divided into two equal aliquots, one of which served as control, while the other was reacted with 0.57 mM DCCD (approx. 100 moles DCCD added/mole COX) as described above. The two aliquots were then dialyzed against 20 mM Tris-Cl, pH 8.5, 1.5% TX-100 at 4°C overnight prior to proteolytic digestion.

For the chymotrypsin concentration dependence of digestion of control and DCCD-treated COX (Wilson and Prochaska, 1990), aliquots of the enzyme were incubated with varying concentrations of α-chymotrypsin-TLCK (Worthington Biochemical, 6.5–40.0 μg) at room temperature. All reactions were quenched after 1 h with 1.92 mM phenylmethylsulfonyl fluoride (in ethanol). COX was collected by ultracentrifugation and SDS–PAGE was performed as described below.

For the time dependence of the limited proteolysis, the enzyme was incubated with α-chymotrypsin-TLCK (COX/chymotrypsin ratio (35 w/w) at room temperature. Aliquots of the enzyme (0.55–0.63 mg aa_3) were removed after 1, 2, or 3 h, and the reaction quenched. SDS–PAGE was performed as described below.

Chemical Cross Linking of COX with SADP

After gel filtration, COX (0.51–1.0 μM aa_3) was reacted with DCCD (0.58 mM) and then reacted with either 0.07 or 0.13 mM *N*-succinimidyl-(4-azidophenyl-1,3′-dithio)-propionate (SADP, dissolved in DMSO)(Pierce) in the dark for 15 min at room temperature. This was followed by irradiation through glass using a Mineralite ultraviolet lamp [3×10^3 erg $(cm^2/s)^{-1}$] for 30–45 min at 0°C for photoactivated cross-link formation. Reactions were quenched with 0.5 M Tris-Cl, pH 8.0. Alternatively, the enzyme was treated with 0.07 or 0.13 mM SADP alone (Estey *et al.*, 1990).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Control and chemically modified COX was collected by ultracentrifugation at 4°C overnight at 192,000 × *g*, in a buffer containing 20 mM Tris-Cl, pH 7.5, and 250 mM sucrose. SDS–PAGE was performed as described by Fuller *et al.* (1981).

Iodoacetyl–Biotin Labeling of Control and DCCD-Modified COX

COX (1.88–2.69 μM aa_3) was incubated with TX-100 and then reacted with either 0.1 or 0.4 mM DCCD as described above. After DCCD treatment, the enzyme was dialyzed against 20 mM $Na_2B_4O_7$, pH 8.3, 90 mM KCl, 0.1% TX-100 and then reacted at room temperature for 4 h with 0.72 mM iodoacetyl–LC–biotin (dissolved in DMSO; Pierce). To quench the reaction, all samples were dialyzed overnight against 20 mM $Na_2B_4O_7$, pH 8.3, 90 mM KCl, 0.1% TX-100 at 0°C.

Avidin-Horseradish Peroxidase Binding to Biotinylated Control and DCCD-Modified COX

Avidin–HRP (5 μM, Pierce) was incubated with unreacted, biotinylated control and biotinylated DCCD-treated COX (7 moles COX/avidin–HRP) at room temperature for 15 min. The mixtures were dialyzed against 5 mM $KHPO_4$, pH 8.0, 0.1% TX-100 and then chromatographed over a horse heart cytochrome *c* affinity column to remove non-specifically bound avidin–HRP. The enzymes (0.45–1.97 μM) were then concentrated using Amicon Centricon 30 or Centricon Plus-20 concentrators and aliquots of each sample were added to 0.8 ml of 3,3′, 5,5′-tetramethylbenzidine base (TMB-ELISA, Life Technologies Inc.) to determine the amount of avidin–HRP. Color formation was monitored spectophotometrically at $A_{450\ nm}$. The observed specific activity of stock avidin–HRP aliquots was from 1.98×10^{10} to 2.67×10^{10} Abs units/s mol. The amount of avidin–HRP bound to COX was determined from the concentration of avidin–HRP determined from the measured initial rates and the avidin–HRP specific activity. COX concentration was determined by absorbance spectroscopy.

SDS–PAGE of Biotinylated Control and DCCD-Treated COX

Biotinylated COX samples were collected by ultracentrifugation as described above. SDS–PAGE

was performed using the system of Dreyfuss *et al.* (1984), modified with 6 M urea in both the 4% acrylamide stacking and 15% acrylamide separating gels. After electrophoresis, the subunits were transferred to nitrocellulose using a semidry apparatus (LKB Multiphor II) and a three-buffer system: the anode buffers were 0.3 M Tris-Cl, pH 10.4, 20% methanol, and also 25 mM Tris-Cl, pH 10.4, 20% methanol. The cathode buffer was 40 mM ϵ-amino-*n*-caproic acid, pH 9.4, 20% methanol. The transfer was complete after 1 h mA at room temperature using a constant current of 0.8 mA/cm^2.

After staining for protein using Ponceau S, the blot was cut into strips for probing with different antibodies. The blots developed with anti-COX and anti-subunit III were first blocked with three washes of 5% nonfat dry milk in TBS. After an overnight incubation at room temperature with antibody diluted in milk–TBS, the blots were then incubated for 2 h at room temperature with a 1:2500 dilution of goat anti-rabbit IgG-alkaline phosphatase conjugate (GIBCO). For blots developed with avidin–alkaline phosphatase, the blot was blocked for 60 min at room temperature in 3% gelatin–TBS, and then incubated for 2 h at room temperature in a 1:500 dilution of avidin–alkaline phosphatase (Bio-Rad) in 1% gelatin–TBST. All rinsed blots were developed using 0.3 mg/ml NBT and 0.15 mg/ml BCIP in 100 mM Tris-Cl, pH 9.5, 0.5 mM $MgCl_2$, and 100 mM NaCl. The reaction was stopped using 20 mM Tris-Cl, pH 8.0, 5 mM EDTA.

RESULTS

Subunit III of COX is a highly conserved subunit in the heme oxidase super-family, yet, to date, the role of subunit III of COX functioning is unresolved. Previous work has shown that DCCD, a known covalent inhibitor of proton translocation in ATP synthase (Fillingame, 1975), blocked proton pumping in COX by covalently binding to Glu90 in subunit III (Casey *et al.*, 1980; Prochaska *et al.*, 1981). The goal of this study was to assess if the DCCD modification of subunit III caused a conformational change in subunit III (Musser *et al.*, 1993). The conformational change in subunit III induced by DCCD could provide a putative mechanism for the previously observed inhibition of proton pumping in the enzyme induced by DCCD.

DCCD Labeling of Subunit III of COX Modifies the Migration of Subunit III on SDS–PAGE

COX was dispersed in TX-100, chromatographed using a Sepharose 2B column, reacted with various concentrations of DCCD for 1 h at room temperature, and then run on SDS–PAGE (Fig. 1). Our previous work showed that subunit III was the primary binding site for DCCD in detergent-solublized COX at all stoichiometries of DCCD added/COX that are presented in Fig. 1. [See Prochaska *et al.* (1981) for a complete discussion.] As the concentration of DCCD was increased in Fig. 1, the migration of subunit III changed to a slower moving form on the SDS–PAGE gel, suggesting that DCCD was covalently bound to the oxidase. The change in migration of subunit III was saturated at 133 moles DCCD added per mole of COX (lane 3). At higher concentrations of DCCD (lanes 4–7), other subunits of the enzyme did not change their migration nor did any new bands appear on the gel, indicating that little or no intersubunit cross linking was induced by DCCD. This result suggests that DCCD treatment did not denature the enzyme extensively under the conditions of our experiments. Additional evidence was that the maximum inhibition of electron transfer activity observed was only 35% at the highest DCCD concentration tested (lane 7, 0.62

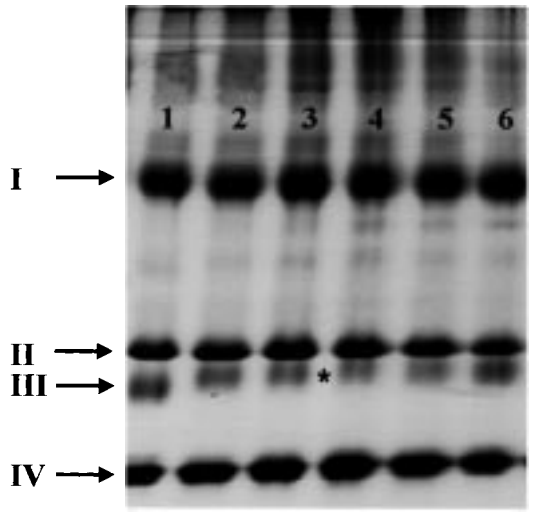

Fig. 1. The concentration dependence of DCCD modification of COX on SDS–PAGE. COX dispersed in TX-100 was reacted with varying concentrations of DCCD, pelleted by ultracentrifugation, and run on SDS–PAGE (Fuller *et al.*, 1981). Lane 1 is control oxidase; lanes 2–6 contain oxidase reacted with DCCD (in mM) 0.06, 0.12, 0.31, 0.50, and 0.62, respectively. Subunit nomenclature is that of Kadenbach *et al.* (1983).

mM DCCD). Also, the reduced absorbance spectrum of the DCCD-treated enzyme was unperturbed at the high concentrations of DCCD. In subsequent experiments, we used concentrations of 133 moles DCCD added/COX or greater.

Limited Proteolysis of Control and DCCD-Modified COX

Having observed that DCCD binding changes the migration of subunit III on SDS–PAGE, we investigated the effect of DCCD modification on the lability of subunit III to limited proteolysis. Previous work has shown that chymotrypsin completely digests subunits III, VI*a*, and *b*, of COX at pH 8.5, without modifying other subunits (Malatesta and Capaldi, 1982; Zhang *et al.*, 1984; Puettner *et al.*, 1985; Wilson and Prochaska, 1990). If the conformation of subunit III was changed by DCCD modification, then we would expect changes in the rate (either an increase or a decrease) of digestion of subunit III by chymotrypsin in the DCCD-modified COX.

The lability of subunit III in control and DCCD treated COX to limited proteolysis was first studied using different chymotrypsin/COX stoichiometries. Figure 2A shows the effect of chymotrypsin concentration dependence on the subunit composition of control and DCCD-treated oxidase at a single time point (1 h). Subunits III, VI*a* and *b* [nomenclature of Kadenbach *et al.* (1983)] were digested to different extents in the chymotrypsin-treated lanes (lanes 3–8). Subunit III in the lanes containing DCCD-treated enzyme (lanes 4, 6, and 8) was more completely digested than in the lanes containing control enzyme (lanes 3, 5, and 7) for all chymotrypsin concentrations tested. For example, in lane 6 of Fig. 2A, subunit III displays a decreased staining intensity compared to lane 5 [COX/chymotrypsin ratio of 42 (w/w)]. Similar results are obtained when comparing lanes 3 and 4 (COX/chymotrypsin ratio of 20), as well as lanes 7 and 8 (COX/chymotrypsin ratio of 71). These results were quantitated using scanning densitometry (Sigma Gel version 1.0) by measuring Coomassie blue staining intensity of subunit III. The results showed subunit III of the DCCD-treated COX was 15–37% more labile to chymotrypsin digestion, thus indicating that DCCD binding changes the conformation of subunit III and exposes more sites for proteolytic digestion.

The time dependence of digestion of subunit III by chymotrypsin after DCCD treatment was also investigated (Fig. 2B), using a COX/chymotrypsin ratio of 35 (w/w). The reaction times were 1 h for lanes 3 and 4, 2 h for lanes 5 and 6, and 3 h for lanes 7 and 8. DCCD treated COX is in the even-numbered lanes of Fig. 2B, while control COX is in the odd-numbered lanes. For all time points, subunit III of the DCCD-modified enzyme exhibited an increased rate (22–52%) rate of digestion compared to subunit III of the control enzyme (as determined by scanning densitometry). After 3 h of incubation with chymotrypsin (lane 8), additional subunits of COX were also digested. These results show that DCCD binding to subunit III not only changes its conformation to make it more labile to proteolysis, but also increases the susceptibility of the other subunits to chymotrypsin digestion.

DCCD Modification of Subunit III Inhibits Intersubunit Cross-Linking Patterns of COX Induced by SADP

To further substantiate whether the conformation of subunit III is changed by DCCD, the enzyme was first treated with DCCD and then cross linked using the heterobifunctional cross-linking reagent SADP (Estey *et al.*, 1990; Estey and Prochaska, 1993). SADP has two reactive ends; one end has limited specificity, reacting with α and ϵ amino groups, and the other end, an azido group, has a relatively nonspecific reactivity with many amino acids, forming a nitrene after photoactivation (Richards and Brunner, 1980). Estey *et al.* (1990) have shown that SADP reacts with COX and extensively cross links subunit III. The reagent also produced new bands appearing on SDS–PAGE, indicating intersubunit cross linking within the enzyme (Estey and Prochaska, 1993).

COX was reacted with either SADP alone (as a control) or with DCCD followed by SADP at the same concentration as the control, to evaluate if DCCD prevented intersubunit cross linking induced by SADP. Figure 3 (lane 3) shows that after SADP treatment, subunit III is cross linked to other subunits of the enzyme and is almost entirely absent from the SDS–PAGE gel. Similar amounts of COX are loaded onto each lane of Fig. 3. When the oxidase was first modified with DCCD and then subjected to the same SADP treatment, subunit III migrated in the gel to a similar position observed for the DCCD modified subunit III (see lanes 4 and 5 compared to lane 2 of Fig. 3). Furthermore, the staining intensity of the subunit III band was appreciably darker (as determined by scan-

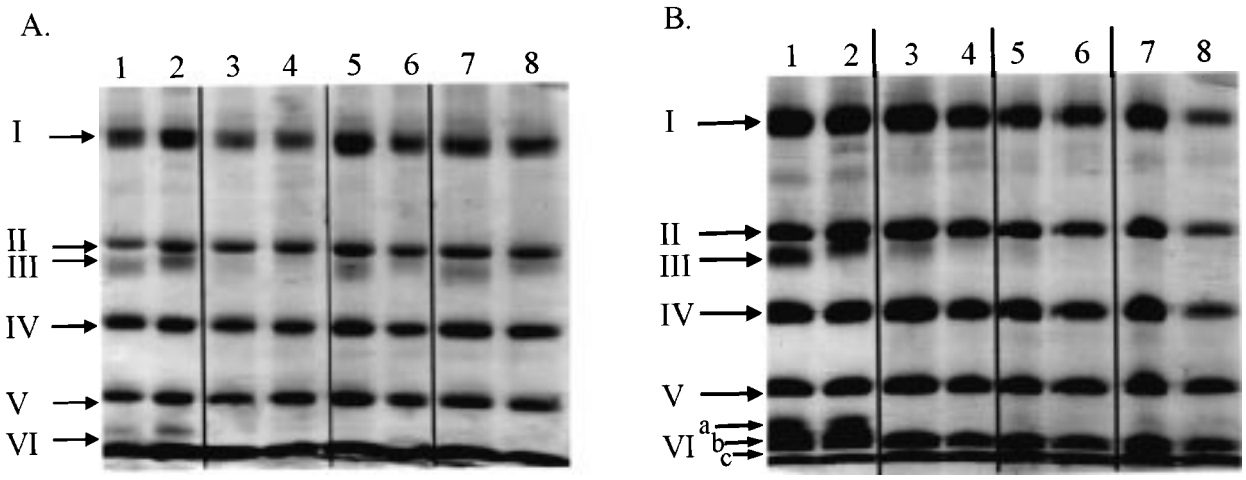

Fig. 2. The chymotrypsin concentration dependence (A) and time dependence (B) of limited proteolysis of control and DCCD-treated COX. COX was dispersed in TX-100, treated with DCCD, and then the DCCD-treated and control COX was digested with α-chymotrypsin as described in the Methods section. (A) Concentration dependence for the chymotrypsin digestion. Lane 1 is untreated oxidase; lane 2 is DCCD-treated oxidase. COX in lanes 3–8 was subjected to chymotrypsin digestion for 1 hr. Lanes 4, 6, and 8 were treated with 0.57 mM DCCD, while lanes 3, 5, and 7 are control COX. Lanes 3 and 4 were treated with a COX/chymotrypsin ratio of 20 (w/w); lanes 5 and 6 were treated with a ratio of 42; and lanes 7 and 8 were treated with a ratio of 71. Scanning densitometry showed that subunit III in lanes 4, 6, and 8 had 15, 37, and 27% less Coomassie Blue stain than in lanes 3, 5, and 7, respectively. (B) Time dependence for chymotrypsin digestion is presented using a COX/chymotrypsin ratio of 35 (w/w). Lanes 1 and 2 are control and DCCD-treated oxidase. Lanes 3–8 were treated with chymotrypsin for different times. Lanes 4, 6, and 8 were treated with 0.57 mM DCCD, while lanes 3, 5, and 7 were control COX. In lanes 3 and 4, 1 h of digestion was used; lanes 5 and 6, 2 h; lanes 7 and 8, 3 h. The lability of the other subunits in oxidase to chymotrypsin digestion after DCCD modification is exhibited in lane 8. Subunit III in lanes 4, 6, and 8 had 22, 52, and 46% less Coomassie Blue stain than in lanes 3, 5, and 7, respectively.

ning laser densitometry) when compared to COX treated with SADP alone [compare lanes 4 (+DCCD) and 3 (−DCCD) in Fig. 3]. Using a higher SADP concentration, a similar protection effect against cross linking was induced by DCCD [Fig. 3, lanes 5 (+DCCD) and 6 (−DCCD)]. This protection against SADP cross linking by DCCD suggests that DCCD binding to subunit III of COX changes the conformation of the subunit to inhibit intersubunit cross linking.

The Effect of DCCD on the Incorporation of Iodoacetyl-Biotin into COX

Subunit III of COX contains two cysteine residues, Cys115 and -218 (Anderson *et al.*, 1982) with Cys115 being surface exposed. Cys115 is very reactive with the water-soluble sulfhydryl reagent, iodoacetamide (Malatesta and Capaldi, 1982; DiBiase and Prochaska, 1985) with as much as 90% of the reagent bound to COX at Cys115. We tested to determine if DCCD binding changes the reactivity of subunit III of COX with biotinylated iodoacetamide. After COX or COX pretreated with DCCD was reacted with iodoacetyl–biotin for 4 h, the treated enzymes were then incubated with avidin conjugated to horseradish peroxidase (HRP) to detect iodoacetyl–biotin incorporation.

Table I shows the effect of DCCD concentration on the incorporation of iodoacetyl–biotin into COX. Although the control contained no iodoacetyl–biotin, avidin–HRP bound nonspecifically to COX. A cytochrome *c* affinity column was used to separate most of the nonspecifically bound avidin–HRP from COX. After elution from the column, the biotinylated enzymes were assayed spectophotometrically to determine the amount of avidin bound. Knowing the initial concentration of avidin–HRP and the amount added to each sample, the specific activity of the avidin–HRP was determined. The specific activity was used to determine the concentration of avidin in each sample.

Table I shows that a low concentration of DCCD (0.1 mM) induced a 1.67-fold increase in avidin bound to COX compared to control enzyme treated with iodoacetyl–biotin. When the DCCD concentration was increased to 0.4 mM, avidin binding increased 3.6-fold. This change in reactivity was also dependent on

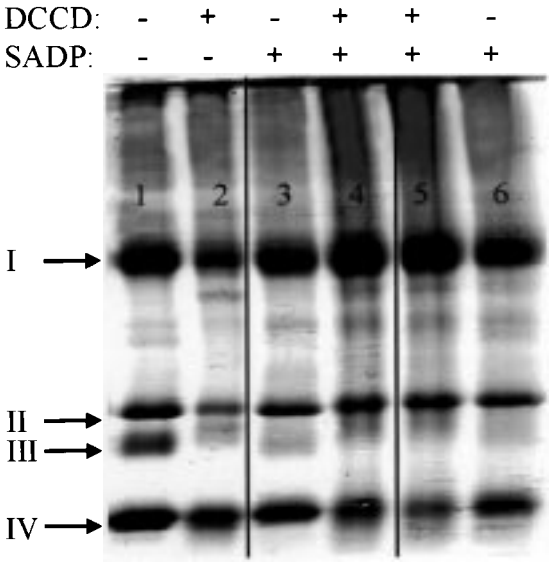

Fig. 3. DCCD modification inhibits SADP cross linking of COX. COX was first modified by DCCD and then subjected to SADP treatment or was treated with SADP alone. Lane 1 is control COX; lane 2 contains 0.62 mM DCCD alone; lane 3 has 0.07 mM SADP alone; lane 4 contains 0.58 mM DCCD and 0.07 mM SADP; lane 5 contains 0.57 mM DCCD and 0.13 mM SADP; lane 6 contains 0.13 mM SADP.

iodoacetyl–biotin concentration (data not shown). The results in Table I suggest that DCCD binding to subunit III changes its conformation to allow more iodoacetyl–biotin labeling of the enzyme.

Table I. Effect of DCCD Pretreatment on the Incorporation of Iodoacetyl–Biotin into Cytochrome *c* Oxidase

Treatment[a]		Avidin/COX after Cyt. *c* affinity column[b]	Fold increase in avidin binding[c]
Iodoacetyl–biotin (mM)	DCCD (mM)		
0	0	0.046 ± 0.001	—
0.072	0	0.076 ± 0.006	1
0.072	0.1	0.127 ± 0.009	1.67
0.072	0.4	0.276 ± 0.027	3.61

[a] All treatments were performed as described in the Methods section.

[b] The avidin/COX ratios after cytochrome *c* affinity column purification were calculated by determining the concentration of avidin and COX within each sample. The concentration of avidin was determined by calculating the observed specific activity (1.98×10^{10} to 2.67×10^{10} Abs/s mol) of avidin. The concentration of COX was determined spectrophotometrically.

[c] Fold increases in avidin binding were calculated by using values from control COX treated with iodoacetyl–biotin.

Quantitation of Iodoacetyl–Biotin Binding to COX Using Alkaline-Phosphatase-Conjugated Avidin on Nitrocellulose

The increased avidin binding to COX induced by DCCD could be attributed to either a direct increase in incorporation of biotinylated acetamide into subunit III, an increased incorporation of the reagent into the entire COX molecule due to extensive conformational changes induced by DCCD, or, quite simply, an increase in nonspecific avidin binding to COX. We determined this by first labeling the control and DCCD-treated enzyme with iodoacetyl–biotin and then measuring the amount of biotinylated acetamide incorporated into the subunits of COX (after SDS–PAGE) on nitrocellulose blots using alkaline phophatase-conjugated avidin. Figure 4 shows (from left to right) a nitrocellulose blot that was developed with antibodies to COX (to locate subunits of COX) (lanes 1–5), antibodies to subunit III (to locate subunit III) (lanes 6–10), and alkaline phosphatase-conjugated avidin (to localize biotinylation) (lanes 11–14). Lane 11 is control enzyme, which was not treated with iodoacetyl–biotin. Lane 12 contains COX treated with iodoacetyl–biotin alone, while lanes 13 and 14 contain

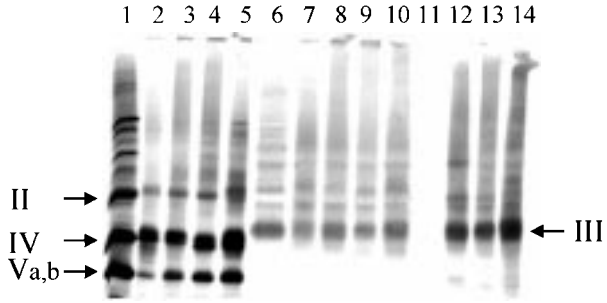

Fig. 4. Detection of the iodoacetyl–biotin-labeled subunits of control and DCCD-treated COX with alkaline phosphatase-conjugated avidin. COX was treated with DCCD as described in the Methods Section. Control and DCCD-treated COX were reacted with iodoacetyl–biotin and were collected by ultracentrifugation. After SDS–PAGE and transfer to nitrocellulose, the blots were sliced into three strips; one strip was developed with anti-COX (1:2500 dilution, lanes 1–5), the second with anti-subunit III (1:100 dilution, lanes 6–10), and the third with avidin conjugated with HRP (lanes 11–14). The first and second strips were washed and then incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase. All strips were then developed. All lanes contain 12 μg of COX. Lanes 1 and 6 contain untreated COX. Lanes 2, 7, and 11 contain control COX. Lanes 8 and 12 contain COX reacted with iodoacetyl–biotin, whereas lanes 9 and 13 and 10 and 14 contain COX that was pretreated with DCCD (0.1 and 0.4 mM, respectively) and then reacted with iodoacetyl–biotin.

COX pretreated with 0.1 mM and 0.4 mM DCCD prior to reaction with iodoacetyl–biotin. The staining intensity in lanes 13 and 14 (Fig. 4) is more intense than that in lane 12 (iodoacetyl–biotin alone), suggesting that DCCD labeling in COX modifies subunit III's reactivity with iodoacetyl. Figure 4 also shows that the increased avidin binding to COX in Table I is due to increased iodoacetyl–biotin labeling in subunit III.

DISCUSSION

In an attempt to delineate the mechanism of DCCD-induced inhibition of proton pumping in COX (Casey *et al.*, 1980; Prochaska *et al.*, 1981), we employed a series of biochemical approaches where first, the enzyme was modified with DCCD and then was subjected to different chemical treatments. Our goal was to investigate if DCCD binding to subunit III of COX causes conformational changes within the subunit to induce the previously observed inhibition of proton pumping. If the conformation of subunit III or subunit III's chemical environment was altered by the covalent binding of the bulky cyclohexyl groups, then subunit III should exhibit different chemical properties to biochemical reagents. In support of our hypothesis, subunit III of DCCD-treated COX was more labile to limited proteolysis with chymotrypsin, less reactive with the heterobifunctional cross-linking reagent, SADP (an indicator of intersubunit interactions), and more reactive with the water-soluble sulfhydrl reagent, iodoacetyl–biotin.

Our results show that when Glu90 is modified with DCCD, there are large changes in the conformation of subunit III. This could be due to helical interactions within the subunit in the structure of the enzyme being disturbed (see PDB file 1OCC for the structure of bovine COX). The binding of the bulky DCCD at Glu90 may disrupt a hydrogen bond (3.0 Å) between Glu90 and the conserved His207 in helix VI, causing a change in the interaction of helices III and VI of subunit III. This displacement of the hydrogen bond could cause the other helices of subunit III to move away from helices I and II in subunit III.

Another hydrogen bond, which may be disrupted after DCCD binding, is between His103 (helix III) in subunit III and Asp227 (helix VIII) in subunit I (a distance of 2.8 Å). The structure of the enzyme shows that there is a cholate molecule (presumably a phospholipid molecule *in vivo*) intercalated between these two amino acid residues. Mutagenesis experiments in *Rhodobacter sphaeroides* COX subunit I have shown that polar residues in helix VIII influence the activity and subunit structure of the active site (Hosler *et al.*, 1996). When the conformation of subunit III is modified, the phospholipid bridge that bonds helix III of subunit III to helix VIII of subunit I could be influenced, which could affect the activity of the oxidase.

An additional hydrogen bond that could be disturbed upon DCCD binding to subunit III is His71 and Glu64 of subunit III and Arg96 of subunit I. There are phospholipid headgroups in the structure that interact with His71 and Glu64 of subunit III and Arg96 of subunit I. This phospholipid contact may act as an allosteric effector by stabilizing the enzyme in a particular conformation (Gennis, 1989; Sandermann, 1978; Kolbe *et al.*, 2000) that is conducive to proton pumping, especially since this site is near the opening of the proposed D-channel (Fetter *et al.*, 1995; Iwata *et al.*, 1995; Tsukihara *et al.*, 1996; Yoshikawa *et al.*, 1998). DCCD modification of subunit III could disturb these interactions and affect the translocation of protons.

Thus, modification of COX by DCCD likely disrupts hydrogen bonds within subunit III and at the subunit I–III interface, which disturb helical interactions between the subunits of the enzyme. The loss of these interactions results in an enzyme complex that loses proton-pumping efficiency. Our experiments confirm the work of Musser *et al.* (1993) who showed that a major conformational change occurs in subunit III when fluorescent carbodiimides are bound at Glu90. Musser *et al.* (1993) also suggested that subunit III could serve as an allosteric effector and regulate the allosteric interactions between subunits I and II necessary for the redox linkage. If this is true, then our assessment that the inhibition of proton pumping induced by DCCD is being caused by a conformational change in subunit III is likely to be correct.

Additional evidence (Haltia *et al.*, 1994) for conformational coupling between subunit III and I is provided by the genetic deletion of subunit III in *P. denitrificans* COX. The deletion of subunit III perturbs the binuclear center (in subunit I), which causes a loss of electron transfer activity as a function of enzyme turnover (suicide inactivation). More recently, similar results have been shown in *Rhodobacter sphaeroides* using biochemical depletion of subunit III (Bratton *et al.*, 1999). Furthermore, the inactivated enzyme exhibits decreased accessibility of the binuclear center to exogenous ligands, an accumulation of reduced heme

a during enzyme turnover, and a partial loss of Cu_B of the binuclear center. Thus, subunit III prevents suicide inactivation by maintaining the structural integrity of the binuclear center.

Further evidence of a strong interaction between subunit III and I is suggested by a mitochondrial myopathy in humans caused by a DNA deletion in subunit III (amino acids 92–97). There is no COX activity *in vivo* (Hoffbuhr *et al.*, 2000) in this deletion. The location of the deletion in the structure of the enzyme is near the subunit I–III interface, suggesting that subunit I–III interactions are required to stabilize the assembled complex. Finally, point mutations in subunit III have been created in *P. denitrificans* COX that were designed to mimic other known myopathies. The expressed mutations, when assayed, showed decreased proton-pumping efficiency, emphasizing that modifications in subunit III affect subunit I activities (Mather and Rottenberg, 1998).

In summary, DCCD binding in subunit III changes the conformation of the subunit by disrupting hydrogen bonds between helices and subunits. In addition, it may modify phospholipid contact with the subunits of the enzyme. Although the function of the phospholipid on oxidase activity is not yet clear, investigation into the interaction of these phospholipids on oxidase activity should be pursued. Since DCCD binding changes the interaction of the subunits of the oxidase, it is likely that subunit III plays a regulatory role, not a participatory role, in the proton-pumping activity of the enzyme.

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