Journal of Bioenergetics and Biomembranes, Vol. 30, No. 5, 1998

Exogenous Ubiquinol Analogues Affect the Fluorescence of NCD-4 Bound to Aspartate-160 of Yeast Cytochrome *b*[1]

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Received July 14, 1998

Previously, we reported that the carboxyl-reacting reagent DCCD, and its fluorescent derivative NCD-4 binds covalently to aspartate-160 localized in amphipathic helix *cd* of the CD loop connecting membrane-spanning helices C and D of cytochrome *b* (Wang *et al.*, 1995). We have investigated the fluorescent properties of NCD-4 to probe possible changes in the *cd* helix resulting from the binding of exogenous ubiquinol analogues to the bc_1 complex. Preincubation of the bc_1 complex with the reduced substrate analogues, DQH_2, DBH_2, and Q_6H_2 resulted in 20–40% increase in the fluorescence emission intensity of NCD-4 and a 10–20% increase in the binding of [^{14}C]DCCD to the bc_1 complex. By contrast, preincubation with the oxidized analogues DQ, DB, and Q_6 resulted in a 20–40% decrease in the fluorescence emission intensity of NCD-4 and a 20–40% decrease in the binding of [^{14}C]DCCD to the bc_1 complex. Moreover, addition of the reduced ubiquinols to the bc_1 complex preincubated with NCD-4 resulted in a blue shift in the fluorescence emission spectrum. In addition, incubation of the cytochrome bc_1 complex reconstituted into proteoliposomes with both reduced and oxidized ubiquinol analogues resulted in changes in the quenching of NCD-4 fluorescence by CAT-16, the spin-label probe that intercalates at the membrane surface. These results indicate that the addition of exogenous ubiquinol to the bc_1 complex may result in changes in the *cd* helix leading to a more hydrophobic environment surrounding the NCD-4 binding site. By contrast, preincubation with the inhibitors of electron transfer through the bc_1 complex had no effect on the binding of NCD-4 to the bc_1 complex or on the fluorescent emission spectra, which suggests that the binding of the inhibitors does not result in changes in the environment of the NCD-4 binding site.

KEY WORDS: Mitochondria; conformation; proton translocation; cytochrome bc_1 complex; fluorescence quenching; ubiquinol–cytochrome-*c* reductase; NCD-4; DCCD.

INTRODUCTION

Ubiquinol–cytochrome-*c* oxidoreductase, the cytochrome bc_1 complex, catalyzes electron transport from ubiquinol (QH_2) to cytochrome *c* coupled to the translocation of protons across the membrane (reviewed by Brandt and Trumpower, 1994; Trumpower and Gennis, 1994). Similar bc_1 complexes are

[1] Abbreviations used: DCCD, *N, N'*-dicyclohexylcarbodiimide; NCD-4, *N*-cyclohexyl-N'-[4-(dimethylamino)naphthyl]carbodiimide; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CAT-16, 4-(*N, N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6, 6-tetramethylpiperidine-1-oxyl iodide; 12-DSA, 12-doxylstearic acid; DQ, duroquinone; DQH_2, duroquinol; DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; DBH_2, reduced DB; Q_6, coenzyme Q_6; Q_6H_2, reduced coenzyme Q_6; HQNO, hydroxyquinoline *N*-oxide.

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an integral part of the electron transport chain of mitochondria and chloroplasts (as the b_6f complex), as well as aerobic and photosynthetic bacteria. All the bc_1 complexes, including the *bf* complex of chloroplasts, contain three subunits with electron transfer components: cytochrome *b* that contains 2 noncovalently bound heme groups, cytochrome c_1 (or cytochrome *f* in chloroplasts) that contains a covalently bound heme group, and an iron–sulfur protein, that contains a [2Fe–2S] cluster. Cytochrome *b*, a hydrophobic integral membrane protein (Beattie *et al.*, 1981), contains a low potential heme (b_L) localized near the positive side of the membrane and a high potential heme (b_H) localized near the negative side of the membrane. The bc_1 complex contains two distinct binding sites for ubiquinone, designated as Q_o and Q_i involved in QH_2 oxidation and Q reduction, respectively, and in concomitant proton translocation (Brandt and Trumpower, 1994).

The recently published crystal structure of the cytochrome bc_1 complex from beef heart mitochondria has revealed that two bc_1 complex monomers interact in the crystal to form a dimer with a twofold axis of symmetry (Xia *et al.*, 1997). The location of the two hemes of cytochrome *b* within the intermembrane region was determined by binding studies with two specific inhibitors of electron transfer in the complex. Antimycin A, a specific inhibitor of electron flow from heme b_H to ubiquinone, was located in the crystal in a pocket next to heme b_H. Myxothiazol, a specific inhibitor of electron flow from ubiquinol to the iron–sulfur center and to heme b_L, was located in the crystal in a pocket within cytochrome *b*, midway between the iron of heme b_L and the [2Fe–2S] cluster of the iron–sulfur protein. The presence of eight membrane-spanning helices in cytochrome *b*, predicted earlier from hydropathy plots (Beattie, 1993; Brasseur, 1988), mutagenesis studies (Colson, 1993; Howell *et al.*, 1987; Gennis *et al.*, 1993; Brasseur *et al.*, 1996), and biochemical experiments (Yun *et al.*, 1991; Beattie *et al.*, 1994), was observed in the crystal structure of the bc_1 complex. In addition, the crystal structure indicates that the CD loop connecting transmembrane helices C and D contains two short helices cd_1 and cd_2 that form a hairpin localized just above the myxothiazol binding pocket, the presumed site of quinol binding to Q_o, on cytochrome *b* (Xia *et al.*, 1997). The involvement of the CD loop in the quinol binding site had been suggested earlier, based on photoaffinity labeling studies of cytochrome *b* with arylazido derivatives of benzoquinone, which were bound to cytochrome *b* in the region indicated by the dashed line in Fig. 1 (He *et al.*, 1994). In addition, several mutations leading to decreased activity of the bc_1 complex and/or resistance to antibiotics that bind to the Q_o site also mapped in this region of cytochrome *b* (Brasseur *et al.*, 1996).

In a previous study (Wang *et al.*, 1995), we reported that the carboxyl-reacting reagent DCCD and its fluorescent derivative NCD-4 bind covalently to aspartate-160 of cytochrome *b*. This conclusion was based on the observation that radiolabeled DCCD was bound in nearly a 1:1 ratio to the bc_1 complex of yeast mitochondria (Beattie and Clejan, 1982) where it was exclusively localized on cytochrome *b* (Beattie *et al.*, 1984). Moreover, exhaustive tryptic digestion or cyanogen bromide cleavage of radiolabeled cytochrome *b* isolated from the bc_1 complex incubated with [^{14}C]DCCD revealed the presence of a single peptide containing radioactive DCCD obtained from each treatment (Wang *et al.*, 1995). Sequencing of the tryptic peptide revealed that aspartate-160, localized in the cd_2 helix of the CD loop of cytochrome *b*, was the only acidic amino acid present in this peptide suggesting that this amino acid residue is the site of DCCD and NCD-4 binding. Moreover, both DCCD and NCD-

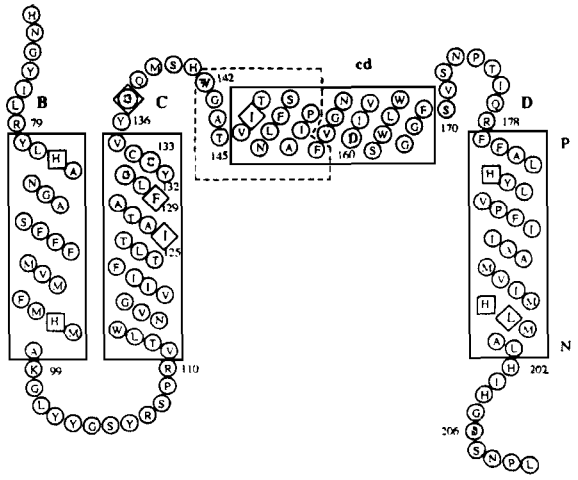

Fig. 1. A model of loop CD of cytochrome *b*. The crystal structure of the cytochrome bc_1 complex has indicated that the *cd* alpha helix is actually bent back to form a hairpin (Xia *et al.*, 1997). The site of NCD-4 binding on aspartate-160 is depicted in bold, the positions of the histidines that bind the two hemes of cytochrome *b* as squares, the sites of mutations that affect electron transfer activity depicted by open letters in dark circles, and the sites of mutations leading to resistance to antibiotics that inhibit electron flow at center Q_o as diamonds (Brasseur *et al.*, 1996). The amino acids included within the box delineated by the dashed line represent a hydrophobic ubiquinone -binding domain identified on beef heart cytochrome *b* (He *et al.*, 1994).

4 inhibited proton translocation in a cytochrome bc_1 complex reconstituted into proteoliposomes without significant inhibition of electron transfer (Beattie and Villalobo, 1982) suggesting that the CD loop of cytochrome *b*, and specifically helix cd_2, may play a role in proton movements in the cytochrome bc_1 complex (Beattie, 1993).

The close proximity of aspartate-160 in the cd_2 helix to the quinol binding site noted in the crystal structure (Xia *et al.*, 1997) prompted us to examine the effects of the exogenous ubiquinol analogues as well as inhibitors of electron transfer through the bc_1 complex on the fluorescence of NCD-4 bound to the bc_1 complex. An increase in the fluorescence intensity coupled with a blue shift in the fluorescence emission spectrum of NCD-4 was observed in the presence of the reduced ubiquinol analogues, Q_6H_2 or DQH_2, to the bc_1 complex. Addition of the oxidized ubiquinol analogues, Q_6, DB, and DQ, blocked the subsequent binding of both NCD-4 and DCCD to the bc_1 complex without any effect on the fluorescence emission spectrum. By contrast, the addition of specific inhibitors of electron transfer through the bc_1 complex had no effect on the fluorescence observed upon addition of NCD-4 to the complex.

EXPERIMENTAL PROCEDURES

Materials

DCCD was obtained from Schwarz-Mann; NCD-4 and the following spin labels were obtained from Molecular Probes Inc., Eugene, Oregon: CAT-16 and 12-DSA; DQ, DB, Q_6, and antimycin were obtained from Sigma. Myxothiazol and stigmatellin were obtained from Boeringer-Mannheim. MOA-stilbene was a generous gift from Dr. Gebhard von Jagow. All of the other chemicals were of the highest purity available commercially.

Preparation of the Cytochrome bc_1 Complex, Labeling with NCD-4, and Incorporation into Proteoliposomes

The yeast strain, KM91, was grown in a fermentor in a medium containing 2% yeast extract, 1% peptone, 2% galactose, and 0.1% $(NH_4)_2SO_4$ (Bruel *et al.*, 1995a). Mitochondria were isolated after breakage of the harvested yeast in a Bronwill Shaker (Beattie and Marcelo–Baciu, 1991). The cytochrome bc_1 complex was isolated from a submitochondrial suspension by the procedure of Ljungdahl *et al.* (1987) as modified in our laboratory (Beattie and Marcelo–Baciu, 1991) and labeled with NCD-4 (Wang and Beattie, 1993). The NCD-4-labeled bc_1 complex was incorporated into proteoliposomes as described previously for the incorporation of the cytochrome *bf* complex labeled with [^{14}C]DCCD (Wang and Beattie, 1992).

Reduction of Ubiquinone and Ubiquinone Analogues

Ubiquinone (Q_6), the decyl analogue of ubiquinone (DB), and duroquinone (DQ) were reduced in ethanol containing 6 mM HCl by addition of a few grains of sodium borohydride until the yellow color had disappeared. The reduced quinols were extracted into petroleum ether and washed three times with water. The petroleum ether was evaporated under a stream of nitrogen and the reduced quinols dissolved in ethanol containing 6 mM HCl. The final concentration of the oxidized and reduced quinone analogues was determined by published extinction coefficients for each analogue (Zhu and Beattie, 1988). To determine the concentration of Q_6H_2, an aliquot of the reduced solution was allowed to remain at room temperature in the absence of HCl so that the Q_6H_2 would be totally oxidized spontaneously. The Q_6 concentration was then measured using the extinction coefficient described above as an indication of the original concentration of Q_6H_2.

Labeling the Cytochrome bc_1 Complex with [^{14}C]DCCD

The cytochrome bc_1 complex containing 2 nmol of cytochrome *b* was suspended in 3 ml buffer containing 100 mM KCl, 0.2 M sucrose, and 5 mM K-HEPES, pH 7.5, and incubated with the concentrations of the ubiquinol analogues described in Fig. 2 (see legend). After a 10-min preincubation at 15°C, [^{14}C]DCCD in a molar ratio to cytochrome *b* of 40:1 was added to the suspension and the incubation continued for 1 h at the same temperature. After immersion of the reaction tubes in an ice bath to stop the reaction, the suspension was layered on top of 30 ml of 10% sucrose and centrifuged for 3 h at 40,000 rpm in a Beckman Ti 45 rotor (Beattie *et al.*, 1984). The pellet

was collected and the proteins separated and analyzed by SDS-PAGE. The gel was stained with Coomassie blue and the staining intensity of the band corresponding to cytochrome *b* determined by laser scanning densitometry. The cytochrome *b* bands were subsequently cut from the gel, dissolved in H_2O_2, and counted in a scintillation counter. The binding of [^{14}C]DCCD to cytochrome *b* was quantitated by dividing the radioactivity determined as counts/min by the intensity of the Coomassie blue-stained protein peak. The labeling of the control bc_1 complex without substrate analogues was set at 100%.

Fluorescence Experiments

The fluorescence emission spectra were recorded and fluorescence quenching were performed in a Shimadzu RF 5000U spectrofluorometer at 25°C as described previously (Wang and Beattie, 1993). The bc_1 complex, at the concentrations indicated in the legends to the figures, was suspended in 2 ml of buffer containing 100 mM KCl, 200 mM sucrose, and 50 mM K-HEPES, pH 6.5. NCD-4 was added in a molar ratio of 40:1 (NCD-4/cytochrome *b*) and incubated for 1 hr at 15°C. The NCD-4 was added from a 100 mM stock solution in ethanol such that the final concentration of ethanol was less than 1%. To remove unbound NCD-4, the labeling mixture was passed through a Diaflo ultra filter YM 100 membrane followed by washing the complex three times with a solution containing 30 mM Tris-succinate, 0.5% sodium cholate, 30 mM octyl glucoside, and 0.1% soybean lecithin, pH 6.5 (Wang and Beattie, 1993). The fluorescence emission spectrum was recorded after excitation of the suspension with light at 340 nm.

The different oxidized and reduced ubiquinol analogues (DQ, DQH_2, DB, DBH_2, Q_6, and Q_6H_2) were added as 2–6 μl aliquots from an ethanol stock solution to the final concentrations indicated in the legends to the figures to the bc_1 complex (0.34 nmol in 2 ml of medium). After 10 min at 15°C, NCD-4 was added and the incubation continued for the times indicated in the figure legends when the fluorescence intensity was determined. Controls in which equivalent amounts of ethanol were added to the complex had no effect on the fluorescence. In a second approach, the bc_1 complex was incubated with NCD-4, as indicated above, for 60 min prior to addition of the ubiquinol analogues. The fluorescence emission spectrum was determined after 5 min.

The inhibitors of electron transfer in the bc_1 complex (myxothiazol, MOA-stilbene, and stigmatellin, antimycin A, Diuron, and HQNO) were individually added to the suspension containing the bc_1 complex (0.34 nmol of cytochrome *b*) and incubated for 10 min at 15°C prior to addition of NCD-4. The fluorescence intensity of each sample was determined at 10-min intervals. The molar ratio of cytochrome *b*/inhibitor was generally 1:10 to yield a final inhibitor concentration of 1 μM or less.

RESULTS

Effect of Ubiquinol Analogues on the Binding of DCCD and NCD-4 to the Cytochrome bc_1 Complex

Previous studies in our laboratory had indicated that DCCD and its fluorescent analogue, NCD-4, bind covalently to cytochrome *b* at aspartate-160 located in the CD loop connecting transmembrane helices C and D (Beattie, 1993). The crystal structure has revealed that this acidic residue is located in helix cd_2 which covers the myxothiazol binding pocket on cytochrome *b* (Xia *et al.*, 1997). To explore the effects of substrates on the environment of the DCCD and NCD-4 binding site on cytochrome *b*, we have examined the effects of several oxidized and reduced ubiquinol derivatives on the binding of both carbodiimides to the bc_1 complex. Preincubating the complex for 10 min with the reduced ubiquinol analogues (DQH_2, Q_6H_2, and DBH_2) prior to addition of NCD-4, resulted in a 20–40% increase in the fluorescence intensity of bound NCD-4 observed after a 1-h incubation (Fig. 2A). By contrast, preincubating the complex with the oxidized analogues, Q_6, DB, and DQ, resulted in a 20–40% decrease in the fluorescence intensity of bound NCD-4. In parallel experiments, we noted that preincubating the bc_1 complex with the reduced ubiquinol analogues Q_6H_2 and DQH_2 resulted in a 10–20% increase in the binding of [^{14}C]DCCD to the bc_1 complex, while DBH_2 had no effect. In addition, preincubation of the complex with the oxidized analogues resulted in a 20–40% decrease in the binding of [^{14}C]DCCD to the bc_1 complex (Fig. 2B). These results suggest that the preincubation of the bc_1 complex with either the oxidized or reduced ubiquinone derivatives affects the region of the CD helix of cytochrome *b* where the carbodiimides interact resulting in the observed changes in the binding of DCCD or NCD-4 to the complex.

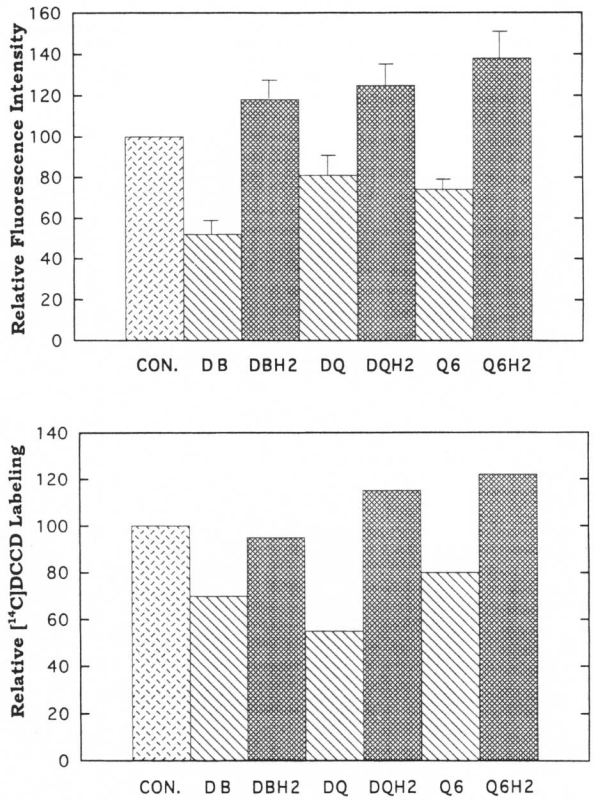

Fig. 2. Effect of ubiquinol analogues on the binding of NCD-4 and [^{14}C]DCCD to the cytochrome bc_1 complex. (A) The bc_1 complex (0.34 nmol of cytochrome *b*) was preincubated with the ubiquinol analogues: 3 (in μm) DQ, 6 (in μm) DQH_2, 3 (in μm) DB, 10 DBH_2, 3 (in μm) Q_6, or 4 (in μm) Q_6H_2 for 10 min prior to the addition of NCD-4 and incubated for 1 h at room temperature. The fluorescence intensity was determined at 430 nm after excitation at 340 nm. The fluorescence of the control without substrates was 200 fluorescent units and was set at 100%. Each point is the average of five determinations ± the standard error of the mean. (B) The bc_1 complex (3.4 nmol cytochrome *b*) was preincubated with the ubiquinol analogues at the concentrations indicated under (A) and then incubated with radiolabeled DCCD for 1 h at 15°C as described in the section on Experimental Procedures. After isolation of the labeled bc_1 complex by centrifugation through 30 ml of 10% sucrose, the labeled proteins were separated by SDS–PAGE. The gel was stained with Coomassie blue and the staining intensity of the band corresponding to cytochrome *b* determined by laser densitometry. The cytochrome *b* band was subsequently cut from the gel and counted in a scintillation counter. The radioactivity in the control band averaged 1000 cpm. The specific activity was determined by dividing the counts/min by the staining intensity of the band corresponding to cytochrome *b*. The control without addition of substrates was set at 100%. Each value is the average of 2 to 4 determinations.

The binding of NCD-4 to the bc_1 complex, as indicated by increasing fluorescence emission intensity, occurred slowly at 15°C, reaching a maximum after 1 h (Fig. 3). The effects of the exogenous quinol analogues on the time course of binding of NCD-4 to the cytochrome bc_1 complex were concentration-dependent. Increasing the concentration of Q_6H_2 from 2 to 4 μM resulted in a 30–40% increase in the fluorescence intensity of NCD-4 (Fig. 3). Similar increases in the fluorescence intensity of NCD-4 were observed with DBH_2; however, it was necessary to use larger concentrations of DBH_2 (5–20 μM) to observe increases in fluorescence. In parallel experiments, increasing the concentration of Q_6 from 2 to 10 μM and DB from 1 to 5 μM resulted in a decrease in the fluorescence intensity due to the binding of NCD-4 to the complex ranging from 20 to 50% (Fig. 3).

In an earlier study, we reported that addition of the reduced ubiquinol (Q_6H_2) to the bc_1 complex previously incubated for 1 h with NCD-4 resulted in a blue shift in the fluorescence emission spectrum (Wang *et al.*, 1995). Similar experiments in which the oxidized ubiquinone analogues (Q_6 and DQ) were added to the NCD-4 treated bc_1 complex did not affect either the intensity of the fluorescence emission or the fluorescence emission spectrum. The addition of DB, however, to the NCD-4 treated complex resulted in minor quenching of the fluorescence due to NCD-4. The decrease in fluorescence due to quenching by DB was approximately 30% of the total decrease in fluorescence observed when DB was added to the bc_1 complex prior to NCD-4. Overall, these results suggest that the decreased fluorescence of NCD-4, observed when the carbodiimide was added to the bc_1 complex previously incubated with the natural quinone analogue, Q_6, can not be explained by fluorescence quenching, while minor fluorescence quenching may have resulted from the addition of DB.

Effect of Ubiquinol Analogues on the Fluorescence Emission Spectrum of NCD-4 Bound to the bc_1 Complex

The changes in fluorescence intensity of NCD-4 bound to the bc_1 complex may also reflect changes in the environment of the probe. To explore this possibility, the different ubiquinol analogues were added to the bc_1 complex after a 1-h incubation with NCD-4 and the fluorescence emission spectrum determined within 5 min. The fluorescence emission spectrum of the control bc_1 complex treated for 1 h with NCD-4 displayed an emission peak at 430 nm after excitation at 340 nm (Fig. 4). After addition of the reduced ana-

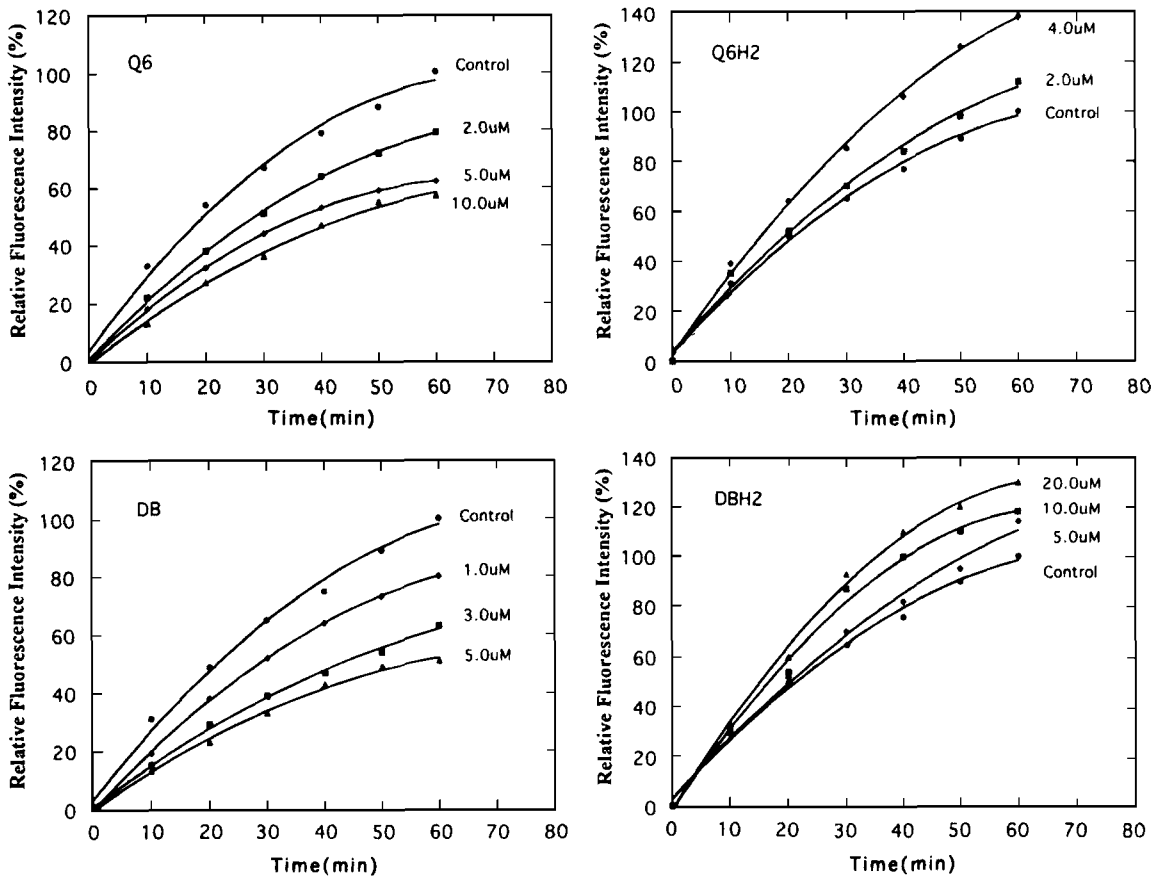

Fig. 3. Time course of NCD-4 binding and concentration dependence of the effects of the oxidized and reduced ubiquinol analogues on the fluorescence of NCD-4 bound to the cytochrome bc_1 complex. The ubiquinol analogues at the concentrations indicated on the figure were preincubated for 10 min with the bc_1 complex (0.34 nmol of cytochrome *b*) prior to addition of NCD-4. Fluorescent measurements were made every 10 min by exciting at 340 nm and measuring the fluorescence at 430 nm after the addition of Q_6, Q_6H_2, DB, and DBH_2 as indicated.

logues, DQH_2, DBH_2, and Q_6H_2, to the bc_1 complex pretreated with NCD-4, a blue shift in the maximum emission spectrum of the NCD-4 treated complex of 3, 5, and 13 nm, respectively, was observed (Fig. 4). The increased fluorescence intensity plus the blue shift of the maximum emission spectrum suggests that the environment of the NCD-4 fluorescent probe has become more hydrophobic upon addition of exogenous reduced ubiquinol analogues. In addition, the observed increase in NCD-4 fluorescence may also arise from an increased binding of the probe, as was observed with [^{14}C]DCCD in the presence of the quinol analogues. In parallel experiments, the oxidized analogues, DQ, DB, and Q_6, were added to the bc_1 complex preincubated for 1 h with NCD-4. Figure 4 indicates that identical emission spectra were observed in the samples preincubated with the oxidized analogues, suggesting that no changes occur in the environment of the probe after addition of these substrates. The decreased fluorescence observed in the presence of exogenous oxidized quinones thus reflects a decreased binding of NCD-4 to cytochrome *b* equivalent to the decreased binding of [^{14}C]DCCD observed under these conditions.

Effect of Inhibitors of Electron Transfer Through the bc_1 Complex on the Fluorescence of NCD-4 Bound to the bc_1 Complex

Inhibitors of electron transfer through the bc_1 complex have been shown to bind to the same, or similar, sites on cytochrome *b* as ubiquinol and ubiquinone (Xia *et al.*, 1997; Link *et al.*, 1993). The fluorescent properties of NCD-4 bound to the bc_1 complex did not change when the complex was preincubated with specific inhibitors of electron transfer through

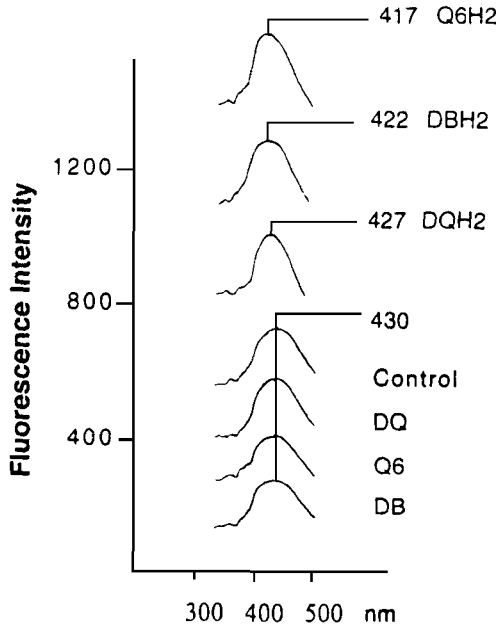

Fig. 4. The fluorescence emission spectra of NCD-4 bound to the cytochrome bc_1 complex preincubated with ubiquinol analogues. The bc_1 complex was incubated with NCD-4 at a molar ratio of 40:1 (NCD-4/cytochrome *b*) for 1 h. At that time the ubiquinol analogues (4 μM Q_6H_2, 10 μM DBH_2, 6 μM DQH_2, 3 μM DQ, 3 μM Q_6 and 3 μM DB) were added to the NCD-4 treated bc_1 complex. After 5 min, the fluorescence emission spectra were measured after excitation at 340 nm.

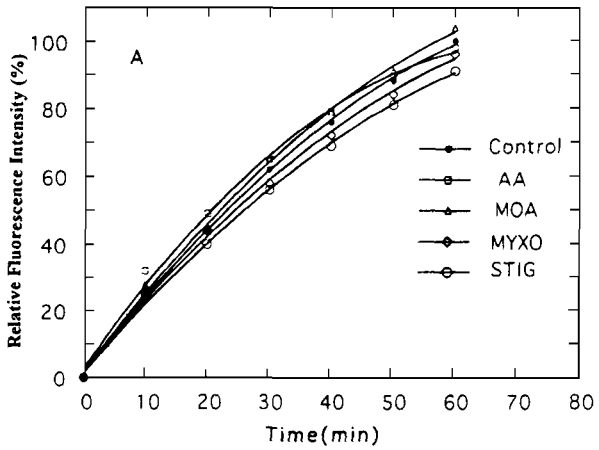

Fig. 5. Effects of inhibitors of electron transfer through the cytochrome bc_1 complex on the binding of NCD-4 to the complex. The inhibitors were added to the bc_1 complex at a final concentration of 1 μM and incubated for 10 min at room temperature prior to addition of the NCD-4. The time course of the increase in fluorescence intensity at 430 nm was determined after excitation at 340 nm as described in the section on Experimental Procedures. AA, antimycin A; MOA, MOA-stilbene; Myxo, myxothiazol; Stig, stigmatellin.

both the ubiquinol oxidizing site, center Q_o, and the ubiquinone reducing site, center Q_i (Fig. 5). The bc_1 complex was preincubated for 10 min with myxothiazol, stigmatellin, and MOA-stilbene, inhibitors at center Q_o, or with antimycin A, HQNO, and Diuron, inhibitors at center Q_i. The inhibitors had no effect on the subsequent time course of the changes in NCD-4 fluorescence. In addition, the fluorescence emission spectrum of the bound NCD-4 did not change in the presence of the inhibitors with the exception of antimycin A, where a 4-nm red shift was observed.

Fluorescence Quenching Experiments

To obtain further information about changes in the NCD-4 binding site resulting from addition of the ubiquinol analogues to the bc_1 complex, paramagnetic fluorescence quenching experiments were performed (Wang *et al.*, 1995; London and Feigenson, 1981; Blatt *et al.*, 1984). Previous quenching studies in our laboratory had suggested that aspartate-160, the NCD-4 binding site in the bc_1 complex reconstituted into proteoliposomes, was localized within the membrane at a distance of approximately 2–3Å from the surface (Wang *et al.*, 1995). This conclusion was based on the observation that the fluorescence of NCD-4 was quenched most effectively by the cationic amphiphilic spin label CAT-16, which partitions in the bilayer with the polar group and the spin label at the membrane surface, and by the hydrophobic probe 5-doxylstearic acid (5-DSA) that intercalates near the headgroup region in the bilayer.

In the current study, similar experiments have been performed with the bc_1 complex reconstituted into proteoliposomes and incubated with the different ubiquinol analogues. The spin-label quenchers used in this study included CAT-16, the amphiphilic probe and the doxyl derivative of stearic acid, 12-DSA, a lipophilic spin-label probe with the doxyl group located on carbon-12 of the fatty acid. As reported earlier (Wang *et al.*, 1995), CAT-16 was a more effective quencher than 12-DSA of the fluorescence observed in the control bc_1 complex labeled with NCD-4 (Fig. 6A, B). Addition of the reduced quinol substrate, Q_6H_2 or DBH_2 to the bc_1 complex resulted in increased quenching by the amphiphilic probe CAT-16, while addition of the oxidized substrates Q_6 and DB resulted in decreased quenching by this probe. The quenching by the 12-DSA probe was unaffected by the addition of the substrates with the exception of DB, which significantly decreased the quenching observed.

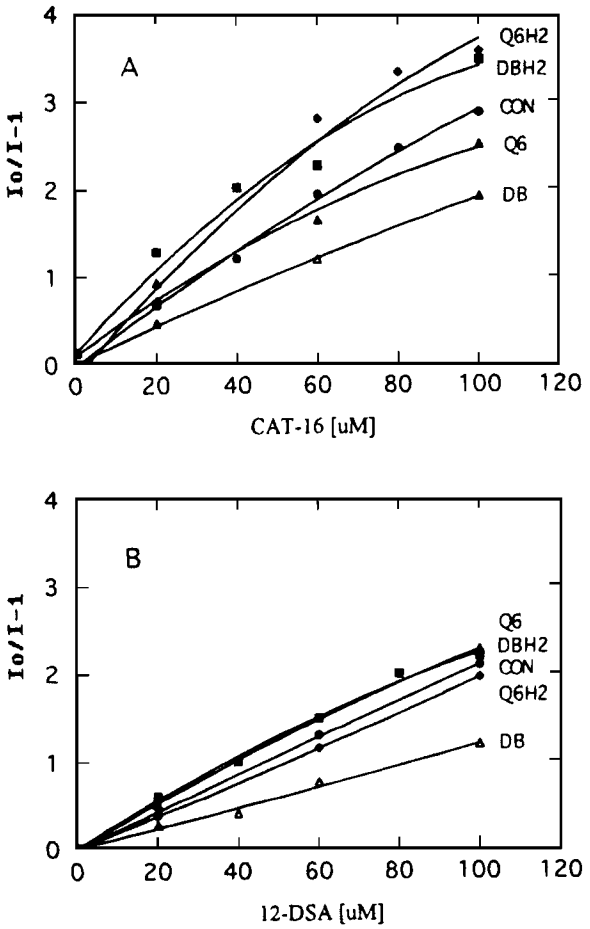

Fig. 6. Fluorescence quenching of NCD-4 labeled cytochrome bc_1 complex in the presence of ubiquinol analogues by spin-label probes. The NCD-4 labeled bc_1 complex was incubated with the ubiquinol analogues, DB, DBH_2, Q_6, and Q_6H_2 at a final concentration of 6 μM (except DB at 2 μM) prior to formation of proteoliposomes. The proteoliposomes were suspended in 2 ml of reaction buffer containing 0.12 μM cytochrome *b* in the bc_1 complex and the quenchers CAT-16 and 12-DSA added to the cuvettes at the final concentrations indicated in the figure. The fluorescent intensity at 430 nm was recorded 5 min after addition of the quenchers. The plot was calculated using the Stern–Volmer equation described earlier (Wang and Beattie, 1993).

These results suggest that in the presence of the reduced quinols, aspartate-160 labeled with NCD-4, may be more exposed to the surface of the membrane. By contrast, in the presence of the oxidized quinones, aspartate-160 may become more deeply buried in the membrane.

DISCUSSION

The experimental results of the current study suggest that the binding of exogenous DQH_2, Q_6H_2, and DBH_2 to the Q_o site of cytochrome *b* results in conformational changes in the environment of NCD-4 bound to aspartate-160 of helix cd_2. The evidence in support of this suggestion includes the increased binding of both NCD-4 and DCCD to the bc_1 complex observed in the presence of the reduced substrates. In addition, a blue shift in the fluorescence emission spectrum of NCD-4 bound to the bc_1 complex was observed as well as an increased fluorescence intensity after addition of the reduced substrates, which suggests that the probe is in a more hydrophobic environment under these conditions. Finally, the amphiphilic spin-label probe that intercalates at the membrane surface, CAT-16, was a more effective quencher of the NCD-4 fluorescence in the presence of the reduced substrates suggesting that the binding of the reduced substrates induced the fluorescent probe to move closer to the membrane interface.

Changes in the environment of the NCD-4 probe bound to aspartate-160 of yeast cytochrome *b* in the presence of exogenous ubiquinols may reflect the role of the CD loop of cytochrome *b* in proton pumping by the bc_1 complex. In earlier studies, the binding of DCCD or NCD-4 to aspartate-160 of cytochrome *b* was shown to result in an inhibition of proton pumping without significant effects on electron transfer through the bc_1 complex (Beattie and Villalobo, 1982; Beattie, 1993; Wang *et al.*, 1995). Similar inhibitions of proton pumping by DCCD have been observed in a *bf* complex isolated from spinach thylakoids (Wang and Beattie, 1991) and the bc_1 complex isolated from *Rhodobacter sphaeroides* (Wang *et al.*, 1998). In both of these complexes, DCCD binds to an acidic amino acid residue located in the CD loop of cytochrome *b*, a further indication that these amino acids may be involved in proton movements at the positive side of the membrane. Our previous studies using spin-label probes had indicated that the binding site for NCD-4 on yeast cytochrome *b* is localized within the membrane but near the surface of the membrane (Wang *et al.*, 1995). This conclusion was based on the preferential quenching of fluorescence by CAT-16, an amphiphilic probe that is located at the membrane–water interface, and by 5-doxylstearic acid that has a spin label located at the C-5 position near the headgroup region of the lipid bilayer. This suggested localization for the NCD-4 binding site on yeast cytochrome *b* is consistent with the localization of the *cd* helix in the crystal structure of the beef heart bc_1 complex in which helices cd_1 and cd_2 are observed to lie along the membrane surface above the myxothiazol binding pocket. The *cd* helixes

may thus act to seal the Q_o site for ubiquinol oxidation from the intermembrane space and thus prevent proton leakage (Xia *et al.*, 1997). Moreover, changes in the conformation of the CD loop upon binding of the reduced substrate may position the proton amino acids localized in this region of cytochrome *b* to facilitate the movement of protons during ubiquinol oxidation.

By contrast, the addition of the oxidized quinone analogues to the bc_1 complex did not result in conformational changes in cytochrome *b* as suggested by their effects on the binding of NCD-4 to the bc_1 complex. Addition of either Q_6 or DB to the bc_1 complex decreased the subsequent binding of both NCD-4 and DCCD to the complex; however, an identical fluorescence emission spectrum of NCD-4 bound to the bc_1 complex was observed after addition of these substrates. These results suggest that the binding of exogenous quinone analogues to center Q_i may block the subsequent entry of the bulky carbodimides through the large cavity observed in the crystal structure of cytochrome *b* (Xia *et al.*, 1997).

The inhibitors of electron transfer, which bind to the Qo binding site, did not affect the fluorescence of NCD-4 covalently bound to aspartate-160 of cytochrome *b*, which suggests that the interaction of these compounds with the quinol binding pocket does not change the environment of the fluorescent probe. Analysis of the crystal structure of the bc_1 complex has indicated separate, but closely overlapping, binding sites for myxothiazol and stigmatellin, which do not pack the Q_o site (Berry, 1997). Moreover, the presence of two ubiquinol molecules in the Q_o site has been demonstrated in the bc_1 complex of the bacterium, *Rhodobacter capsulatus* (Ding *et al.*, 1995). We suggest that exogenous Q_6H_2 and DBH_2 may still bind to the Q_o site in the presence of the inhibitors such that the resulting changes in the *cd* helix are observed in the fluorescence of NCD-4.

A decoupling of electron transfer and proton pumping in the bc_1 has also been described in mutants of yeast cytochrome *b* in which appreciable electron transfer activity of the bc_1 complex is retained despite the inability of these strains to grow on respiratory substrates (Lemesle-Meunier *et al.*, 1993). It should be noted that several of these "uncoupled mutants," such as mutations of glycine-137, tryptophan-142, and threonine-142 are located in the CD loop of cytochrome *b*. Further study of the mutant in which glycine-137 had mutated to a glutamate residue indicated that the energy produced during the redox reactions was not coupled to the formation of a proton gradient across the membrane (Bruel *et al.*, 1995b). In preliminary studies in our laboratory with a bc_1 complex isolated from this G147E mutant, significantly more NCD-4 was bound to cytochrome *b*, perhaps due to the presence of the additional acidic amino acid residue in the hydrophobic environment of the CD loop. In addition, the fluorescence emission maximum of NCD-4 was red-shifted 10 nm suggesting that the environment of the probe in the mutant was less hydrophobic. The substitution of the polar glutamate for glycine at residue 137, located at the start of the CD loop, may lead to a different polarity in the environment surrounding the NCD-4 probe and contribute to the loss of proton-pumping ability in these cells through changes in the pK_a of acidic groups. These decoupling mutations as well as the effects of carbodiimides binding to acidic residues provide additional evidence for the importance of the *cd* helices on proton pumping at center Q_o. Further characterization of various mutants of cytochrome *b* is currently underway to explore the environment of the *cd* helix and its role in proton translocation.

ACKNOWLEDGMENTS

We would like to thank Drs. James Mahaney and Andrew Shiemke for many helpful discussions, Ms. Marilyn Howton for preparation of the cytochrome bc_1 complex, and Dr. Danielle Lemesle-Meunier for her support and encouragement. This study was supported, in part, by a grant from the NIH GM 38433.

REFERENCES

Beattie, D. S., (1993). *J. Bioenerg. Biomemb.* **25**, 233–244.
Beattie., D. S. and Clejan, L. (1982). *FEBS Lett.* **149**, 245–248.
Beattie, D. S., and Marcelo–Baciu, R. M. (1991). *J. Bioenerg. Biomembr.* **23**, 665–678.
Beattie, D. S., and Villalobo, A. (1982). *J. Biol. Chem.* **257**, 14745–14752.
Beattie, D. S., Clejan, L., Chen, Y., Lin, C. P., and Sidhu, A. (1981). *J. Bioenerg. Biomembr.* **13**, 357–372.
Beattie, D. S., Clejan, L., and Bosch, C. G. (1984). *J. Biol. Chem.* **259**, 10526–10532.
Beattie, D. S., Jenkins, H. C., and Howton, M. M. (1994). *Arch. Biochem. Biophys.* **312**, 292–300.
Berry, E. A., personal communication, 1997.
Blatt, E., Chatelier, R. C., and Sanyer, R. C. (1984). *Photochem. Photobiol.* **39**, 477–483.
Brandt, U., and Trumpower, B. L. (1994). *Crit. Rev. Biochem. Mol. Biol.* **29**, 165–197.
Brasseur, G., Saribas, A. S., and Daldal, F. (1996). *Biochim. Biophys. Acta* **1275**, 61–69.
Brasseur, R. (1988). *J. Biol. Chem.* **263**, 12571–12575.

Bruel, C., DiRago, J. P., Slonimski, P. P., and Lemesle-Meunier, D. (1995a). *J. Biol. Chem.* **270**, 22321–22328.
Bruel, C., Manon, S. Guerin, M., and Lemesle-Meunier, D. (1995b). *J. Bioenerg. Biomembr.* **27**, 527–539.
Bruel, C., Wang, Y. D., and Beattie, D. S., unpublished observations.
Clejan L., and Beattie, D. S. (1996). *J. Biol. Chem.* **258**, 14271–14275.
Colson, A. M. (1993). *J. Bioenerg. Biomembr.* **3**, 211–220.
Ding, H., Moser, C. C., Robertson, D. E., Tokito, M. K., Daldal, F., and Dutton, P. L. (1995). *Biochemistry* **34**, 15070–15996.
Gennis, R. B., Barquera, B., Hacker, B., Van Doren, S. R. Arnaud, S., Crofts, A. R., Davidson, E., Gray, D. A., and Daldal, F. (1993). *J. Bioenerg. Biomembr.* **25**, 195–209.
He, D. Y, Yu, L., and Yu, C. A. (1994). *J. Biol. Chem.* **269**, 2292–2298.
Howell, N., Appel, J., Cook, J. P., Howell, B., and Nauswirth, W. W. (1987). *J. Biol. Chem.* **262**, 2411–2414.
Lemesle-Meunier, D., Brivet-Chevillotte, P., DiRago, J. P., Slonimski, P. P., Bruel, C., Tron, T., and Forget, N., (1993). *J. Biol. Chem.* **268**, 15626–15631.
Link, Th. A., Haase, U., Brandt, U., and von Jagow, G. (1993). *J. Bioenerg. Biomembr.* **25**, 221–232.
Ljungdahl, P. O., Pennoyer, J. D., Robertson, D. E., and Trumpower, B. L. (1987). *Biochim. Biophys. Acta* **891**, 227–241.
London, E., and Feigenson, G. W. (1981). *Biochemistry* **20**, 1932–1938.
Trumpower, B. L., and Gennis, R. B. (1994). *Annu. Rev. Biochem.* **63**, 675–716.
Wang, Y. D., and Beattie, D. S. (1991) *Arch. Biochem. Biophys.* **291**, 363–370.
Wang, Y. D., and Beattie, D. S. (1992). *Biochemistry* **31**, 8455–8459.
Wang, Y. D., and Beattie, D. S. (1993). *Biochemistry* **32**, 9586–9591.
Wang, Y. D., Howton, M. M., and Beattie, D. S. (1995). *Biochemistry* **34**, 7476–7482.
Wang, Y. D., Obungu, V. H., and Beattie, D. S. (1998). *Arch. Biochem. Biophys*, **352**, 193–198.
Xia, D., Yu, C. A., Kim, H., Xi, J. Z., Kachurin, A. M., Zhang, L., Yu, L., and Deisenhofer, J. (1997). *Science* **277**, 60–66.
Yun, C. H., Van Doren, S. R., Crofts, A. R., and Gennis, R. B. (1991). *J. Biol. Chem.* **266**, 10967–10973.
Zhu, Q. S., and Beattie, D. S. (1988). *Biochim. Biophys. Acta* **934**, 303–313.