Binding of Dicyclohexylcarbodiimide to Aspartate-155 or Glutamate-166 of Cytochrome b_6 in a Cytochrome *bf* Complex Isolated from Spinach Thylakoids†

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ABSTRACT: In a recent study [Wang & Beattie (1991) *Arch. Biochem. Biophys. 291*, 363–370], we reported that dicyclohexylcarbodiimide (DCCD) inhibited proton translocation in the cytochrome *bf* complex reconstituted into proteoliposomes and was bound selectively to cytochrome b_6. To establish the site of binding of DCCD on cytochrome b_6, the cytochrome *bf* complex labeled with [^{14}C]DCCD was selectively digested with chymotrypsin and trypsin. A 17-kDa fragment containing radioactive DCCD and the heme moiety was obtained after chymotrypsin digestion, while a 12.5-kDa fragment containing both radioactive DCCD and the heme moiety was obtained after trypsin digestion, suggesting that the site of DCCD binding might be on aspartate-140, aspartate-155, or glutamate-166. Extensive digestion of cytochrome b_6 isolated from a [^{14}C]DCCD-labeled cytochrome *bf* complex with trypsin followed by isolation and sequencing of two radioactive peptides obtained revealed that DCCD is bound at either residue aspartate-155 or residue glutamate-166 localized in amphipathic extramembranous helix IV. In addition, the cytochrome *bf* complex labeled with [^{14}C]DCCD was reconstituted into liposomes and digested with trypsin. Three fragments of 9.3, 10.5, and 11.5 kDa were obtained, suggesting that the four-helix model for the topography of cytochrome b_6 in the membrane is correct.

The cytochrome *bf* complex, an intrinsic membrane-bound protein complex localized in the thylakoid membranes of chloroplasts of higher plants, mediates electron flow between photosystems I and II (Cramer et al., 1987). As electrons move from plastoquinol through the cytochrome *bf* complex to plastocyanine, protons are translocated across the thylakoid membrane into the lumen of the thylakoids such that an electrogenic proton gradient is generated across the membrane (Hauska et al., 1983). A similar function is contributed by the cytochrome bc_1 complex of the mitochondrial membrane which transfers electrons from ubiquinol to cytochrome *c* with the translocation of protons in the opposite direction across the mitochondrial membrane to the cytosolic side of the inner membrane (Hauska et al., 1983).

Recently, we reported that dicyclohexylcarbodiimide (DCDD),[1] the well-established carboxyl-modifying reagent, inhibited the proton-translocating device in the cytochrome *bf* complex isolated from spinach chloroplasts reconstituted into proteoliposomes (Sprague et al., 1988). Concentrations of DCCD which block electrogenic proton movements in the cytochrome *bf* complex reconstituted into proteoliposomes had a minimal inhibitory effect on the duroquinol:cytochrome *c* oxidoreductase activity of either the soluble or the reconstituted *bf* complex (Wang & Beattie, 1991). These results paralleled previous studies indicating that DCCD blocked proton translocation in the cytochrome bc_1 complex isolated from yeast mitochondria reconstituted into proteoliposomes without significant effect on ubiquinol:cytochrome *c* oxidoreductase in either the complex or the rat liver mitochondria (Beattie & Villalobo, 1982; Clejan & Beattie, 1983; Clejan et al., 1984). The lack of inhibition of electron flow by DCCD suggests that the primary effect of DCCD is on the proton-translocating device of both the cytochrome *bf* and the cytochrome bc_1 complexes. Subsequently, it was shown in this laboratory that radioactive DCCD binds selectively to cytochrome *b* of the bc_1 complex (Beattie et al., 1984) and to cytochrome b_6 of the *bf* complex (Wang & Beattie, 1991), suggesting that these proteins provide a similar function in proton translocation.

The goal of the current study was to determine the actual binding site for DCCD on cytochrome b_6, as this information would indicate which amino acids play a role in proton pumping. DCCD has been shown to bind covalently to aspartate or glutamate residues localized in hydrophobic residues of proteins involved in proton translocation such as the proteolipid of the F_1/F_o proton-translocating ATPase (Fillingame, 1980; Yoshida et al., 1981, 1982), the plasma membrane H^+ ATPase of *Neurospora crassa* (Sussman et al., 1987), subunit III of cytochrome *c* oxidase (Casey et al., 1980; Prochaska et al., 1981), and the transhydrogenase of bovine heart mitochondria (Wakabayaski & Hatefi, 1987). The binding site of [^{14}C]DCCD on cytochrome b_6 has been analyzed through selective proteolytic digestion of the soluble cytochrome *bf* complex followed by identification of peptides containing radioactive DCCD and staining for heme. Sequence analysis of the peptides obtained after exhaustive digestion of isolated cytochrome b_6 labeled with [^{14}C]DCCD in the *bf* complex suggested that DCCD is bound to either aspartate-155 or glutamate-166 localized in the extramembranous amphipathic helix IV (Figure 1A). In addition, the digestion of the cytochrome *bf* complex labeled with DCCD and incorporated into proteoliposomes provided further evidence for the four-helix-folding model for cytochrome b_6 in the membrane suggested by the data of Szczepaniak and Cramer (1990).

EXPERIMENTAL PROCEDURES

Preparation of the Cytochrome bf Complex. The cytochrome *bf* complex was isolated from spinach chloroplasts by the procedure of Hurt and Hauska (1981) as described previously (Hauska, 1986; Wang & Beattie, 1991).

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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride).

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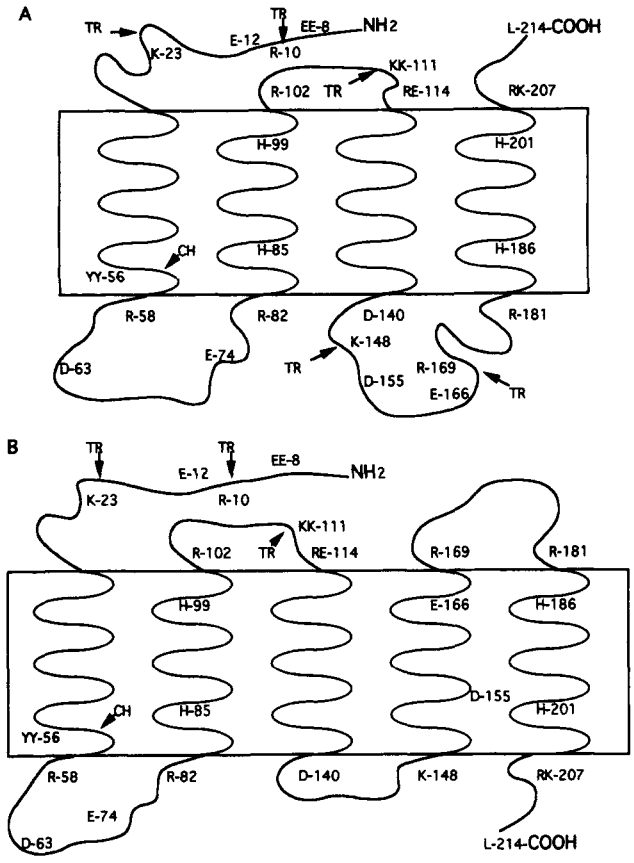

FIGURE 1: Four- (A) and five-helix (B) models of cytochrome b_6 in the membrane bilayer. The charged residues indicated on the diagram represent possible sites for digestion by trypsin (K and R) or chymotrypsin (F, Y, and W) and for the binding of DCCD (D and E).

Labeling of the Cytochrome bf Complex with [^{14}C]DCCD. The cytochrome *bf* complex isolated from spinach chloroplasts was suspended in a medium containing 100 mM KCl/5 mM K-Hepes, pH 7.5, and incubated with [^{14}C]DCCD (30 nmol of DCCD/nmol of cytochrome b_6) dissolved in methanol at a molecular ratio of 30 nmol of DCCD/nmol of cytochrome b_6 at 12 °C for 1 h. Excess radiolabeled DCCD was removed by filtration through a Diaflo ultrafilter YM 100 membrane and washing the complex 3 times with a solution containing 30 mM Tris–succinate acid, 0.5% sodium cholate, 30 mM octyl glucoside, and 0.1% soybean lecithin, pH 6.5. The eluate was counted until the counts had decreased to background levels.

Incorporation of the Cytochrome bf Complex into Liposomes. Proteoliposomes were prepared as described by Hurt et al. (1982). In this procedure, 100 μL of *bf* complex containing 0.3 mg of [^{14}C]DCCD-labeled complex was mixed with 50 mg of soybean lecithin in a 1-mL solution containing 30 mM octyl glucoside, 0.5% sodium cholate, and 30 mM Tris–succinate, pH 6.5. The mixture was sonicated in a small tube with a Branson sonicator using a microtip with full output at position 1 for 4 min. The tube was cooled in an ice/water bath during sonication. The suspension of proteoliposomes was further incubated for 1 h at 4 °C.

Proteolytic Digestion of Soluble Cytochrome bf Complex. A labeled *bf* complex (0.5 mg) was incubated at room temperature for 30 min with 0.1 mg of chymotrypsin in a solution containing 25 mM MOPS/100 mM KCl, pH 7.0. The digestion was stopped by addition of 3 μL of 100 mM PMSF to the incubation mixture. The digestion sample was analyzed by SDS–PAGE. Alternately, the labeled *bf* complex was mixed with trypsin at a ratio of 1/80 to 1/100 (trypsin/protein) and incubated at room temperature for 10 min. The reaction was terminated by adding soybean trypsin inhibitor at a ratio of 1/2 (trypsin/inhibitor). The digested sample was analyzed by SDS–PAGE.

Isolation of DCCD-Labeled Cytochrome b_6 from the bf Complex and Protease Digestion of the Isolated b_6. Cytochrome b_6 was isolated from the *bf* complex previously labeled with [^{14}C]DCCD by electroelution and digested with trypsin in a ratio of 1/20 (trypsin/protein) at 4 °C for 48 h. The digested sample was analyzed by SDS–PAGE in a 15% acrylamide gel with a 0.75-mm thickness without glycine. After electrophoresis, one lane of digested cytochrome b_6 was stained with Coomassie blue, scanned by laser-scanning densitometry, and sliced into 2-mm slices, and each slice was dissolved in 1 mL of 30% H_2O_2 prior to counting in a scintillation counter. The remainder of the gel was blotted electrophoretically to a poly(vinylidene difluoride) (PVDF) protein sequencing membrane in a medium containing 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid]/10% methanol, pH 11.0, with an OWL HEP-1 electrotransfer system. The peptides were sequenced by Dr. John Hempel in the Protein Sequence Facility at the University of Pittsburgh School of Medicine, Pittsburgh, PA.

Proteolytic Digestion of the Cytochrome bf Complex Reconstituted into Proteoliposomes. Proteoliposomes containing the *bf* complex were digested at room temperature for 60 min with trypsin by adding the trypsin solution in the ratio of 1/20 (trypsin/protein) directly into the proteoliposome suspension. The digestion was terminated by adding soybean trypsin inhibitor at a final concentration double the trypsin concentration. The phospholipids were extracted with a 24-fold excess of ice-cold 95% acetone. The pellets were collected by centrifugation for 10 min at 6000 rpm in the SS-34 rotor of the Sorvall centrifuge and washed 3 times with the cold acetone. The final pellet was resuspended in dissociation buffer and analyzed by SDS–PAGE. The gel was stained with Coomassie blue and counted as described above.

Miscellaneous Procedures. In general, SDS–PAGE was performed as described by Laemmli (1970) in gels of 15% acrylamide. To isolate the tryptic peptides of cytochrome b_6, the modified gel system of Schagger et al. (1986) containing Tris–Tricine in the upper buffer and Tris-HCl in the lower buffer was used.

Materials. Radioactive [^{14}C]DCCD, obtained from Amersham (55 mCi/mmol), in toluene was evaporated to dryness under a stream of nitrogen and redissolved in methanol just prior to each experiment. All other chemicals were of the highest purity available commercially.

RESULTS AND DISCUSSION

Previous studies in our laboratory had indicated that the carboxyl-reacting reagent DCCD selectively inhibited proton movements without significantly affecting electron transfer in a cytochrome *bf* complex isolated from spinach thylakoids and reconstituted into proteoliposomes (Wang & Beattie, 1991). Moreover, under identical experimental conditions, radiolabeled DCCD was preferentially bound to cytochrome b_6 in either a soluble or a membrane-bound cytochrome *bf* complex. As a first approach to determining the exact amino acid on cytochrome b_6 to which DCCD is bound, we have utilized selective proteolytic digestion of both the soluble cytochrome *bf* complex and the complex reconstituted into proteoliposomes as well as isolated cytochrome b_6. To identify the putative peptide fragments of cytochrome b_6, we have taken advantage of three known biochemical characteristics

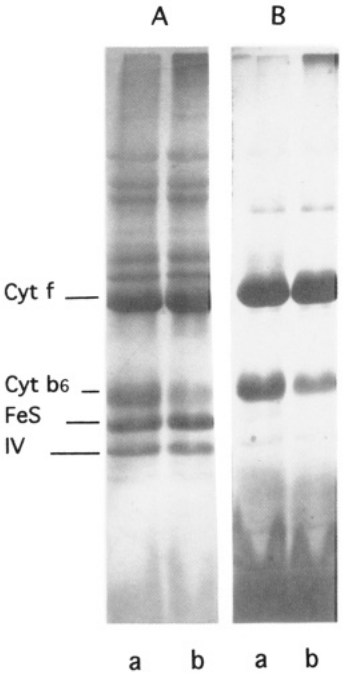

FIGURE 2: Effect of boiling the *bf* complex on the migration of cytochrome b_6. The purified *bf* complex in dissociation buffer was incubated at room temperature for 1 h prior to SDS–PAGE [(A) lane a and (B) lane a]. The *bf* complex in dissociation buffer was heated to 100 °C for 3 min prior to SDS–PAGE [(A) lane b and (B) lane b]. (A) is stained with Coomassie blue. (B) is stained for heme.

of cytochrome b_6, namely, (1) the presence of radioactivity in the peptide due to the binding of [^{14}C]DCCD, (2) the presence of heme indicated by specific staining with benzidine, and (3) the observation that cytochrome b_6 (and its proteolytic digestion products) aggregates upon boiling of the sample prior to electrophoresis. The latter observation is demonstrated in Figure 2. Boiling the cytochrome *bf* complex for 3 min in dissociation buffer prior to SDS–PAGE resulted in the disappearance of the band corresponding to cytochrome b_6 from the gel with the concomitant appearance of a new band at the top of the gel representing aggregated cytochrome b_6 (Figure 2).

Limited digestion of the soluble cytochrome *bf* complex previously labeled with [^{14}C]DCCD with chymotrypsin produced several novel peptides (compare Figure 3A, lanes b and c). One of these proteolytic fragments, named C1, with an apparent molecular mass of 17 kDa contained radioactivity due to the binding of labeled DCCD (Figure 3B), stained with the specific heme stain benzidine (Figure 3B), and disappeared from the gel when the sample was boiled for 3 min prior to SDS–PAGE (Figure 3A, lane c). We conclude from these observations that the 17-kDa peptide is a fragment of cytochrome b_6 cleaved by digestion of the *bf* complex with chymotrypsin. It should be noted that subunit IV of the cytochrome *bf* complex also has an apparent molecular mass of 17 kDa; however, subunit IV does not stain with heme and does not aggregate upon boiling. An examination of the primary sequence of cytochrome b_6 (Figure 1) suggests that a 17-kDa peptide retaining the two hemes would result from the single cleavage of cytochrome b_6 by chymotrypsin at tyrosine-56 or tyrosine-57. The resultant peptide which would extend from the site of cleavage to the carboxyl terminus of cytochrome b_6 contains several acidic amino acids in hydrophobic sequences where DCCD might bind. We feel confident that the 17-kDa peptide is derived from cytochorme b_6, since under these conditions cytochrome *f*, the only other heme-containing protein of the *bf* complex, is essentially resistant

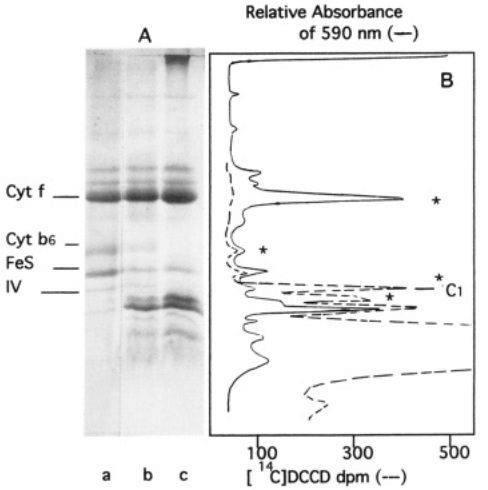

FIGURE 3: Effect of chymotrypsin digestion on a soluble cytochrome *bf* complex previously incubated with [^{14}C]DCCD. (A) Control *bf* complex prior to digestion (lane a), *bf* complex digested with chymotrypsin as described under Experimental Procedures (lane b), and *bf* complex digested with chymotrypsin and then boiled for 3 min prior to SDS–PAGE (lane c). The gel of the chymotrypsin-digested *bf* complex was stained for heme. (B) Densitometric scan of lane b (—) and the radioactivity in 2-mm slices of lane b (- - -). The protein bands that stained for heme are indicated with an asterisk.

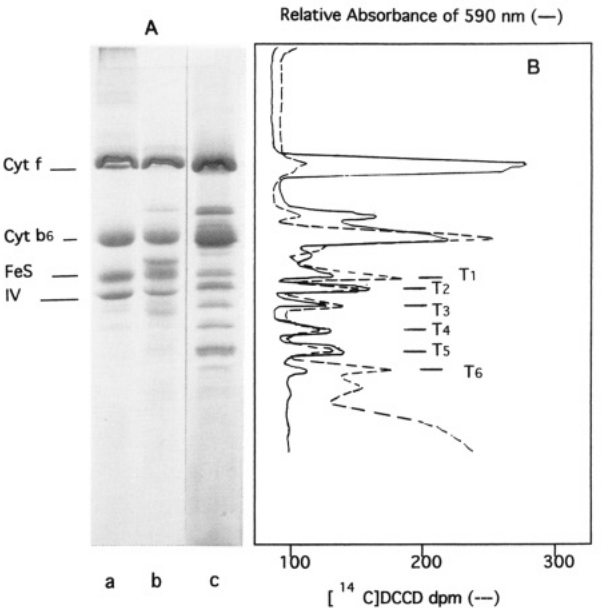

FIGURE 4: Effect of trypsin digestion on the cytochrome *bf* complex. (A) Control *bf* complex prior to digestion (lane a) and *bf* complex digested with trypsin as described under Experimental Procedures (lanes b and c). Coomassie blue-stained (lane b) and specifically stained for heme (lane c). (B) Densitometric scan of lane c (—) and the radioactivity in 2-mm slices of lane b or c (- - -). The bands labeled T1–T6 are the peptide bands that contain both heme and radioactive label.

to chymotrypsin digestion (Figure 3A, lane b) and is not labeled with radioactive DCCD.

Subsequent digestion of the cytochrome *bf* complex previously labeled with [^{14}C]DCCD with trypsin produced six peptides, all of which contained significant radioactivity (Figure 4B, peptides T1–T6). Moreover, each of these peptides stained for heme and disappeared from the gel when the trypsin-digested sample was boiled prior to SDS–PAGE (data not shown). From these three criteria, we have concluded that these six peptides with apparent molecular masses ranging from 19.5 to 12.5 kDa all originate from trypsin digestion of cytochrome b_6. The smallest peptide with an approximate

molecular mass of 12.5 kDa capable of retaining the two hemes would result from the trypsin cleavage of cytochrome b_6 at two sites: arginine-82 and arginine-206 (or lysine-207). The observation that a peptide of this molecular mass is labeled with [^{14}C]DCCD further suggests that the site of binding of radiolabeled DCCD to cytochrome b_6 must be either residues glutamate-114, aspartate-140, aspartate-155, or glutamate-166 as these are the only acidic amino acids in that region of cytochrome b_6 (Figure 1).

To identify the precise labeling site(s) of DCCD on cytochrome b_6, cytochrome b_6 was isolated from a *bf* complex previously labeled with [^{14}C]DCCD and then subjected to exhaustive trypsin digestion at low temperatures. After the digestion, several peptides containing radioactivity were observed; however, the greatest amount of radioactivity was observed in a peptide with an apparent molecular mass of 6.0 kDa (Figure 5A, peptide B2). Partial sequence analysis of this peptide confirmed this assignment (Figure 5, sequence B2). Of the first 20 amino acids in the sequence, 15 were identical to those deduced from the gene sequence of chloroplast b_6 including the first 4 amino acids and the 2 acidic amino acids (D and E). We suggest that trypsin cleaved cytochrome b_6 at lysine-148 and at arginine-206 (or lysine-207) to produce a peptide, B2, with an apparent molecular mass of 6.0 kDa with two potential DCCD binding sites at residues 155 or 166 (Figure 1).

The radiolabeled peptide with the apparent molecular mass of 6.5 kDa was also sequenced (Figure 5A, B1). We suggest that this peptide resulted from a single trypsin cleavage at lysine-148 with the resultant peptide extending to the carboxy terminus of the protein. Six of the first seven amino acids of this peptide are identical to those in the gene sequence including the potential DCCD binding site on aspartate-155. The first amino acid in this peptide could not be accurately determined (Figure 5, sequence B1).

Obtaining sufficient amounts of these peptides after SDS–PAGE and electrophoretic transfer to PVDF membrane proved to be a difficult task, as contamination with other peptides including digestion products of the trypsin itself was observed. Indeed, the analysis of the amino acids released during automated sequencing often revealed glycine and threonine in the third and fourth cycles in addition to the expected threonine and glycine which we suggest represent contaminating peptides. Sufficient sequence homology with the gene sequence for both peptides has lead us to conclude that DCCD binds to either residue 155 or residue 166 on cytochrome b_6.

Two models have been proposed for the topographical orientation of cytochrome b_6 in the thylakoid membrane (Figure 1). In the initial projection, cytochrome b_6 was predicted to contain five hydrophobic membrane-spanning helices (Widger et al., 1984). Recently, this model was revised after analysis of both mean hydrophobicities and hydrophobic moments to predict four membrane-spanning helices for cytochrome b_6 with the original helix IV removed from the membrane into a superficial alignment close to the surface of the membrane (Brasseur, 1988). Recent evidence from Cramer's laboratory (Szczepaniak & Cramer, 1990) has located both the amino and carboxyl termini of cytochrome b_6 on the stromal side of the thylakoid membrane, suggesting that the four-helix model for cytochrome b_6 is correct. To confirm this assignment and to provide further evidence for the site of binding of [^{14}C]DCCD on cytochrome b_6, the *bf* complex reconstituted into proteoliposomes was digested with trypsin. Several peptides were produced after trypsin digestion of the reconstituted complex previously labeled with [^{14}C]-DCCD; however, only three of these peptides with apparent molecular masses of 9.3, 10.5, and 11.5 kDa were radioactive (Figure 6, peptides R1, R2, and R3). We suggest that cytochrome b_6 present in the reconstituted complex was digested by trypsin either at residues arginine-102 and lysine-111 or at residues arginine-114 and arginine-206 or lysine-207 to produce fragments containing 93, 96, and 105 amino acids with the observed molecular masses. Examination of the four-helical model indicates that all of these basic amino acids are on the same side of the membrane. Several potential cleavage sites, lysine-148, arginine-169, and arginine-181, would be shielded from proteolytic cleavage in this model. Moreover, trypsin digestion of cytochrome b_6 in the five-helical model would not result in radioactive peptides with the observed molecular masses, because arginine-169 and arginine-181 are exposed on the same side of the membrane.

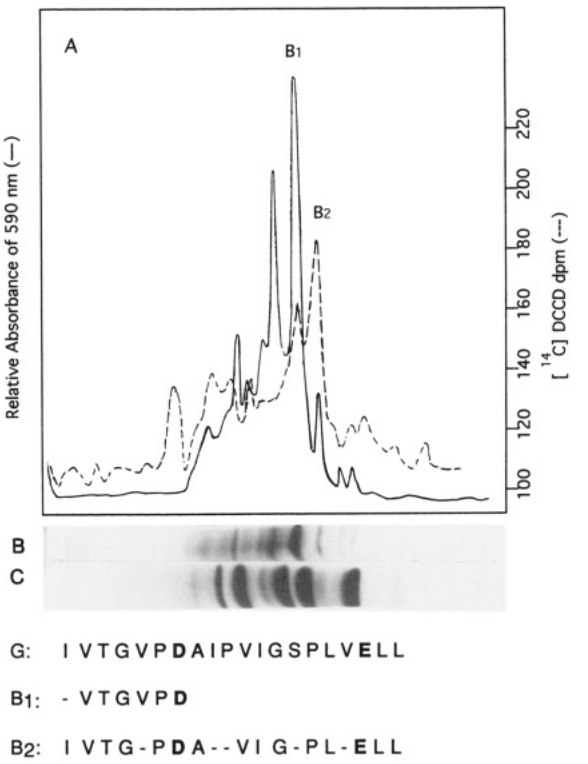

FIGURE 5: Complete digestion of isolated cytochrome b_6 by trypsin. Cytochrome b_6 was isolated by electroelution from a *bf* complex previously treated with [^{14}C]DCCD. The purified cytochrome b_6 was digested by trypsin for 48 h at 4 °C prior to SDS–PAGE in 15% acrylamide in a buffer without glycine (Schaegger et al., 1986). (A) Densitometric trace of cytochrome b_6 after trypsin digestion (—) and the radioactivity in 2-mm slices of the gel shown in lane B (- - -). Coomassie blue-stained gel of cytochrome b_6 after trypsin digestion (lane B). Molecular mass standards of 16.9, 14.4, 8.16, 6.2, and 2.5 kDa reading from left to right (lane C). Sequence of the putative peptide containing radioactive DCCD; deduced from the gene sequence (lane G), partial sequence of the peptide with an apparent molecular mass of 6.5 kDa labeled B1 in the trace in (A) (lane B1); partial sequence of the peptide with an apparent molecular mass of 6.0 kDa containing the most significant radioactivity labeled B2 in the trace in (A) (lane B2). The dashes indicate positions in the sequence where the amino acid identity was ambiguous.

As a control, the soluble cytochrome *bf* complex was digested with trypsin under the same conditions as the *bf* complex reconstituted into proteoliposomes (Figure 6C). After trypsin digestion, no significant radioactive label was present in peptides with molecular masses above approximately 6.0 kDa, suggesting that trypsin-sensitive cleavage sites on cytochrome b_6, arginine-102, lysine-110, lysine-111, arginine-113, arginine-148, arginine-169, and arginine-181, can be digested by trypsin under these conditions.

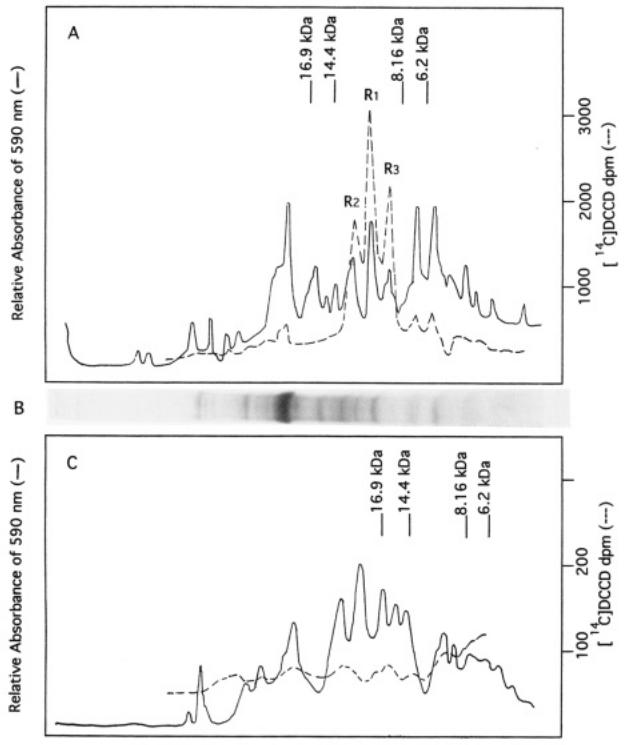

FIGURE 6: Effect of trypsin digestion of the cytochrome *bf* complex reconstituted into proteoliposomes. (A) Densitometric trace of the Coomassie blue-stained gel (—). The gel was sliced into 2-mm pieces, dissolved in H_2O_2, and counted in a scintillation counter (- - -). The three peaks labeled R1, R2, and R3 with apparent molecular masses of 9.5, 10.5, and 11.5 kDa are the peptides containing significant radioactivity. (B) Stained gel of the *bf* complex reconstituted into proteoliposomes after trypsin digestion for 1 h at room temperature. (C) Effect of trypsin digestion on a soluble cytochrome *bf* complex under the same conditions as used for the *bf* complex reconstituted into proteoliposomes (A). Densitometric scan of the Coomassie blue-stained gel (—) and radioactivity in 2-mm slices of the same gel (- - -). The numbers indicate the migration of the standard molecular weight markers.

CONCLUSIONS

The results of the current study suggest that radioactive DCCD binds to either aspartate-155 or glutamate-166 of cytochrome b_6. This conclusion is based on the peptides observed after selective digestion of a soluble cytochrome *bf* complex, isolated cytochrome b_6, or a *bf* complex reconstituted into proteoliposomes with trypsin or chymotrypsin. The retention of radiolabeled DCCD on peptide fragments containing heme provided additional confidence in this assignment. Partial sequence analysis of 6.0- and 6.5-kDa peptides obtained by extensive trypsin digestion of isolated cytochrome b_6 confirmed this conclusion.

It should be noted that the assignment of either aspartate-155 or glutamate-166 as the site of DCCD binding places this site in an extramembranous, yet hydrophobic, helix of cytochrome b_6 and as such has implications for the pathway of protons from the site of quinol oxidation localized within the membrane either to the bulk phase outside the membrane or to a localized proton gradient. Current studies in our laboratory with fluorescent derivatives of DCCD are addressing the implications of this conclusion.

Interestingly, neither aspartate-155 nor glutamate-166 is conserved in cytochrome *b* of mitochondrial bc_1 complexes. An, asparate, however, is present in the same extramembranous hydrophobic helix at residue 160 in cytochrome *b* from yeast, bovine, human, and *Aspergillus* mitochondria (Widger et al., 1984). In our laboratory, we have shown that DCCD blocks proton release in the cytochrome bc_1 complex isolated from yeast mitochondria and binds selectively to cytochrome *b* (Beattie & Villalobo, 1982; Beattie et al., 1984). Recently, we have obtained a single peptide containing radiolabeled DCCD after cyanogen bromide cleavage of cytochrome *b*.[2] The molecular weight of this peptide was consistent with the binding of DCCD to aspartate-160 of cytochrome *b*, suggesting that the acidic amino acid residues present in the extramembranous helix may play a role in the proton conductance pathway.

[2] Beattie and Howton, manuscript in preparation.

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Registry No. DCCD, 538-75-0; aspartic acid, 56-84-8; glutamic acid, 56-86-0.