Aspartate-187 of Cytochrome *b* Is Not Needed for DCCD Inhibition of Ubiquinol: Cytochrome *c* Oxidoreductase in *Rhodobacter sphaeroides* Chromatophores†

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ABSTRACT: *N*,*N*′-dicyclohexylcarbodiimide (DCCD) has been reported to inhibit steady-state proton translocation by cytochrome bc_1 and b_6f complexes without significantly altering the rate of electron transport, a process referred to as decoupling. In chromatophores of the purple bacterium *Rhodobacter sphaeroides*, this has been associated with the specific labeling of a surface-exposed aspartate-187 of the cytochrome *b* subunit of the bc_1 complex [Wang et al. (1998) *Arch. Biochem. Biophys. 352*, 193−198]. To explore the possible role of this amino acid residue in the protonogenic reactions of cytochrome bc_1 complex, we investigated the effect of DCCD modification on flash-induced electron transport and the electrochromic bandshift of carotenoids in *Rb. sphaeroides* chromatophores from wild type (WT) and mutant cells, in which aspartate-187 of cytochrome *b* (Asp^{B187}) has been changed to asparagine (mutant B187 DN). The kinetics and amplitude of phase III of the electrochromic shift of carotenoids, reflecting electrogenic reactions in the bc_1 complex, and of the redox changes of cytochromes and reaction center, were similar (± 15%) in both WT and B187DN chromatophores. DCCD effectively inhibited phase III of the carotenoid bandshift in both B187DN and WT chromatophores. The dependence of the kinetics and amplitude of phase III of the electrochromic shift on DCCD concentration was identical in WT and B187DN chromatophores, indicating that covalent modification of Asp^{B187} is not specifically responsible for the effect of DCCD-induced effects of cytochrome bc_1 complex. Furthermore, no evidence for differential inhibition of electrogenesis and electron transport was found in either strain. We conclude that Asp^{B187} plays no crucial role in the protonogenic reactions of bc_1 complex, since its replacement by asparagine does not lead to any significant effects on either the electrogenic reactions of bc_1 complex, as revealed by phase III of the electrochromic shift of carotenoids, or sensitivity of turnover to DCCD.

The main components of cyclic electron transport in chromatophores of non-sulfur purple bacteria are the photosynthetic reaction center (RC)[1] and cytochrome bc_1 complex. On illumination, the RC reduces ubiquinone and oxidizes cytochrome c_2. The cytochrome bc_1 complex oxidizes ubiquinol and reduces cytochrome c_2, with coupled proton release and uptake, and generates a transmembrane electrochemical gradient of protons, which can be used for ATP synthesis, ion transport, and other kinds of work (*1*). The reaction center (*2*−*6*) and the mitochondrial cytochrome bc_1 complex (*7*−*10*) have been crystallized and their structures solved to 2.5−3 Å atomic resolution.

The proton-motive Q-cycle (*11, 12*, reviewed in ref *13*), widely accepted as the underlying mechanism of cytochrome *bc* (and *bf*) complexes, links electron transfer and vectorial proton transport in an obligatory fashion. However, numerous investigators have reported conditions for steady-state measurement in which this obligate coupling appears to be violated. Among these is the effect of the covalent carboxyl-modifying agent, *N*,*N*′-dicyclohexylcarbodiimide (DCCD). It has been shown that the H^+/e^- ratio can be decreased after modification of the cytochrome bc_1 complex by DCCD (*14*−*19*), or by *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) (*19, 20*). Treatment of many preparations with DCCD results in substantial (>50−60%) loss of proton release from the "o" side, with less than 10−20% effect on the steady-state electron transport by the cytochrome *bc* complex. Susceptible preparations include yeast (*21*), rat liver mitochondria (*22*), beef heart mitochondria (*23*) (complex III), chloroplasts (Cyt b_6f complex) (*24*), and membrane preparations from *Rb. sphaeroides* (*25*). Similar behavior is observed after treatment of bovine cytochrome bc_1 complex with the reagent EEDQ, which modifies buried carboxyl groups (*20*). Bruel et al. (*26*) found a partial decoupling of the cytochrome bc_1 complex of *Saccharomyces cerevisiae* induced by a point mutation at residue 137 of the cytochrome *b* subunit, glycine → glutamate.

The origin of such effects observed under steady-state conditions is not clear, but the chemical modifications are

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[1] Abbreviations: EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; ΔpH, transmembrane pH gradient; RC, photosynthetic reaction center; WT, wild type; B187DN, mutant in which aspartate 187 in cytochrome *b* is replaced by asparagine; PMS, *N*-methylphenazonium methosulfate.

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presumed to occur at carboxyl groups of the protein. Several hypotheses have been introduced to explain the decoupling of electron and proton transport by DCCD (reviewed in ref *13*). It is suggested that DCCD blocks the proton channel(s) conducting the proton generated during ubiquinol oxidation at center "o". This, in turn, forces the proton to move along an artificial pathway through the complex to the other side of the membrane (*13*).

Wang et al. (*25*) showed that isotopically labeled DCCD specifically modifies surface-exposed aspartate-187 of cytochrome *b* in *Rb. sphaeroides*, and they assigned the decoupling effect of DCCD to modification of this amino acid residue. However, as noted by Crofts et al. (*27*), this residue is not close enough to the Q_0 site, or potential H^+ channel connected to the site, to have an obvious functional role. Furthermore, DCCD can modify not only carboxyl groups but also sulfhydryl groups and tyrosines (*18*), and it is possible that the effects of DCCD are the results of cumulative modifications of many amino acid residues in the cytochrome bc_1 complex (*19*). To circumvent these uncertainties of chemical modification, we examined the electrogenic and electron-transfer activities of the cytochrome bc_1 complex in *Rb. sphaeroides* chromatophores from wild type and mutant cells in which aspartate-187 in cytochrome *b* has been changed to asparagine (mutant B187DN).

EXPERIMENTAL PROCEDURES

Growth of Rb. sphaeroides and Isolation of Chromatophores. The wild type and mutant strains of *Rb. sphaeroides* Ga were grown aerobically at 30 °C in the dark in Sistrom's medium, in the presence of kanamycin (20 μg/mL) and tetracycline (1.5 μg/mL). Then cells were grown semiaerobically at 30 °C in Sistrom's minimal medium enriched with yeast extract, (bacto)tryptone and casamino acids (Difco, Detroit, USA) and with 1.5 μg/mL tetracycline as described in Takahashi and Wraight (*28*). Except where indicated, cells were finally grown anaerobically in the light, for one day, prior to harvesting. *Rb. sphaeroides* Ga cells were disrupted by a single pass through a French press at 18 000 psi in the presence of a small amount of DNase. Chromatophores were isolated in 10 mM HEPES (pH 7.5) by differential centrifugation as described elsewhere (*29*).

Site-Directed Mutagenesis. The plasmid pBC9, a pUC9 derivative carrying the *fbc* operon of *Rb. sphaeroides*, was used for mutagenesis of the cytochrome *b* subunit of the cytochrome bc_1 complex (*30*). Residue 187 of the cytochrome *b*-subunit (B187) was altered from Asp (GAC) to Asn (AAC) [mutation $Asp^{B187} \rightarrow$ Asn or B187DN] by oligonucleotide-directed mutagenesis (*31*). Mutation was screened by double-stranded DNA sequencing using the Sequenase kit (Amersham). The *fbc* DNA fragment containing the B187DN mutation was transferred to tetracycline-resistant (Tc^R) plasmid pRK415 (*32*), resulting in the plasmid pRKB187DN. The kanamycin-resistant (Km^R) *Rb. sphaeroides fbc*-deletion strain, BC17C (*30*) was complemented by pRKB187DN through a conjugational mating method (*30*, *33*). The DNA fragment containing wild-type (WT) *fbc* operon was also transferred to pRK415, resulting in plasmid pRKBWT, which was used to complement strain BC17C as WT control.

Modification of Chromatophores by DCCD. Chromatophores were incubated with aliquots of freshly prepared 400 mM DCCD stock solution in ethanol for 40 min at room temperature. Control samples were treated with the ethanol only. After treatment, chromatophores were washed by 50 mM MOPS, 100 mM KCl buffer (10 vol of sample).

Spectrophotometric Determination of Redox Changes of Cytochromes, Photoactive Pigment, and Electrochromic Shift of Carotenoids. Kinetics of cytochromes and the electrochromic carotenoid bandshift were measured with a single-beam kinetic spectrophotometer of local design. Light pulses were delivered by xenon flash (<10 μs half-duration). The redox changes of cytochrome c_1 plus cytochrome c_2 were measured at 551−542 nm. The extinction coefficient used for c_{tot} at 551−542 nm was 20 mM^{-1} cm^{-1} (*34*, *35*). Cytochrome b_h reduction was measured at 561−569 nm in the presence of antimycin, and its concentration was estimated using an extinction coefficient of 20 mM^{-1} cm^{-1} (*12*). The concentration of reaction centers was estimated at 542 nm using an extinction coefficient of 10 mM^{-1} cm^{-1} for P870 (*34*). The electrogenic activity of cytochrome bc_1 complex was monitored by measuring the slow phase (phase III) of the carotenoid electrochromic bandshift at 503 nm.

Molecular Visualization. The X-ray structures were visualized using Rasmol (*36*).

Materials. Antimycin A, DCCD, HEPES, and MOPS were obtained from Sigma Chemical Co. Myxothiazol was obtained from Fluka.

RESULTS

Kinetics of Electrochromic Shift and Electron Transport in WT and B187DN Chromatophores. WT and mutant chromatophores were characterized by measuring the light-induced electrochromic shift and redox reactions of cytochromes *b* and *c*, at low redox potentials ($E_h \approx 100$ mV) when the quinone pool is partially reduced before the flash, as well as at high potentials ($E_h \approx 300-350$ mV), when electron transport in the cytochrome bc_1 complex is induced by ubiquinol. We did not observe any significant differences (<15%) between the two types of chromatophores, either in the electrochromic shift (when normalized to RC concentration) or in the kinetics of cytochromes *b* and *c* reduction.

Figure 1 shows the light-induced electrochromic shift (panel A) and kinetics of light-induced redox reactions of cytochromes *b* (panel B) and *c* (panel C) for the two strains measured at 100 mV. Here the concentrations of bc_1 complex and RCs in WT chromatophores are close to those in the mutant chromatophores (± 15%), and no normalization procedure was applied. Consequently, the small differences observed, which were not systematic, reflect the small degree of stoichiometric variability that exists. These data indicate that the carotenoid bandshift in mutant chromatophores is less than that in WT chromatophores after multiple flashes, but the difference between them does not exceed 15% and the difference on the first flash is negligible. The cumulative effect is almost certainly due to slightly higher decay rate (membrane leakage) in this mutant sample.

The flash-induced carotenoid band shift in chromatophores has been frequently described in terms of three phases. Phases I and II are attributed to reactions in or close to the RC, and phase III is attributed to reactions of the bc_1 complex (reviewed in ref *37*). The total amplitude of phases I and II

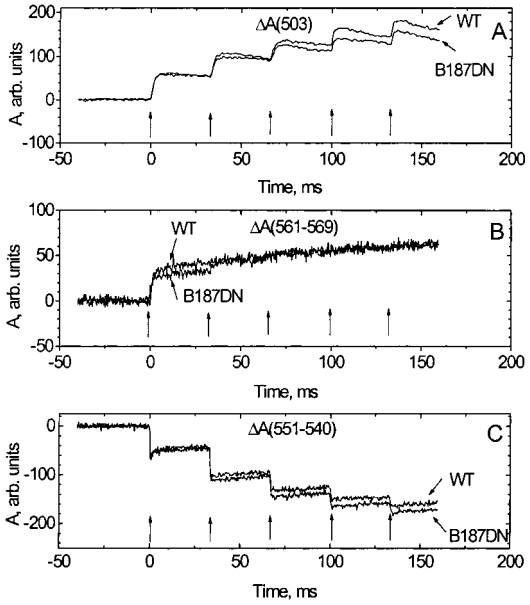

FIGURE 1: Flash-induced kinetics of the electrochromic shift of carotenoids (panel A) and redox reactions of cytochromes *b* (panel B) and *c* (panel C) in the WT and B187DN chromatophores at low redox potential (E_h = 100 mV) under anaerobic conditions. Incubation medium: 50 mM MOPS, pH 7.5, 100 mM KCl plus 5 μM antimycin A in panels B and C only. 2 μM 1,2-naphthoquinone, 2 μM PMS were used as redox mediators, 1 mM Fe(III)EDTA was present as redox buffer. 2 μg/mL gramicidin and 2 μM valinomycin were present in panel B and C to collapse the transmembrane electrochemical potential. Final concentration of cytochrome bc_1 complex within chromatophores was $\approx$1 μM. Each curve is an average of three traces with 60 s dark adaptation between flash series. Both WT and mutant chromatophores were isolated from photosynthetically grown cells.

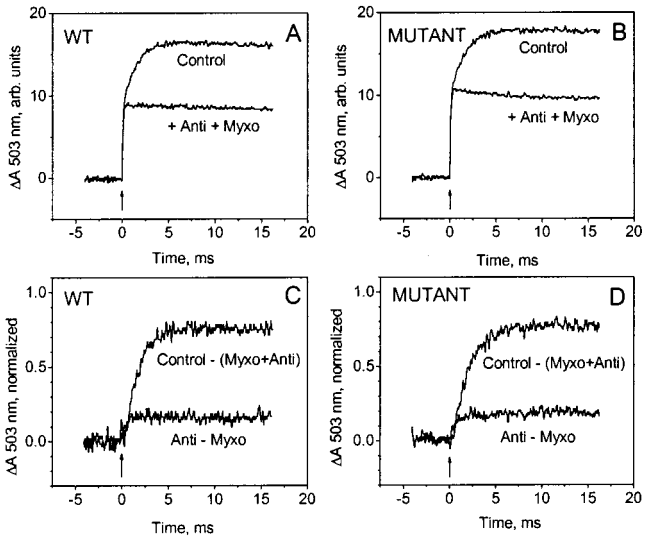

FIGURE 2: Flash-induced electrochromic shift of carotenoids in WT (A, C) and B187DN (B, D) chromatophores in the absence (A, B, upper trace) and presence (A, B, lower trace) of 10 μM antimycin A and 5 μM myxothiazol. Panels C and D, antimycin-sensitive (upper trace) and antimycin-insensitive, myxothiazol-sensitive (lower trace) phases of the electrochromic bandshift in WT (C) and mutant (D) chromatophores. Original traces were normalized at t = 200 μs prior to subtraction to yeild C, D. Incubation medium: 50 mM MOPS, pH 7.5, 100 mM KCl. 2 μM 1,2-naphthoquinone, 2 μM PMS were used as redox mediators, 1 mM Fe(III)EDTA was present as redox buffer (E_h = 100 mV).

of the carotenoid band shift represents transfer of one charge per RC entirely across the membrane and serves as an internal calibration of $\Delta\psi$. The electrochromic carotenoid bandshift is normalized here on the relative amount of reaction centers, which is indicated by the fast rise phase at t = 200 μs (phase I and II).

Figure 2 shows that there is no significant (<15%) difference in either the antimycin-sensitive or myxothiazol-sensitive, antimycin-insensitive electrogenic phases of the bc_1 complex in WT and mutant chromatophores.

Effect of DCCD Modification on the Electrochromic Shift and Electron Transport. Figure 3 shows the effect of DCCD modification of chromatophores on the electrochromic bandshift of carotenoids measured at 503 nm. The DCCD treatment modifies phases I and II (associated with electrochromic events occurring in the reaction center) and phase III of the carotenoid bandshift. In both WT and mutant chromatophores, the effect of DCCD on phase III of the electrochromic shift is similar. At low concentrations (<200 μM) DCCD stimulates the amplitude of phase III and modifies its kinetics (see also Figure 4). The stimulation is probably due to a decrease in the membrane conductivity caused by DCCD binding to F_0 and blockage of the proton flux through F_0F_1 (*38*, *39*). Further increase of the DCCD concentration leads to significant slowing of phase III.

Panels C and D in Figure 3 show phase III alone, which was obtained as the difference between traces with DCCD and traces in the presence of antimycin and myxothiazol (panels A and B). To separate the effect of DCCD on phases I and II and phase III these differences were taken after normalization of trace amplitudes at 0.2 ms.

Treatment of WT and mutant chromatophores with DCCD induced a significant slowdown of the kinetics of phase III of the electrochromic bandshift. We also observed the development of a lag phase in the kinetics of phase III. Figure 4 shows the dependence of the rate constant (single-exponential fit) and amplitude of phase III of the electrochromic shift on DCCD concentration in WT and mutant chromatophores. Both dependencies are almost identical for WT and mutant chromatophores. For DCCD concentrations from 100 to 1200 μM, there is about a 10-fold slowing of phase III. However, there is no significant change in the amplitude. The similarity of the effect of DCCD in WT and B187DN chromatophores strongly indicates that Asp^{B187} is not the primary source of the DCCD effect observed in WT chromatophores.

Figure 5 shows the effect of DCCD on the kinetics of flash-induced cytochrome (c_2 + c_1) rereduction in WT and mutant chromatophores. In both cases, the rate of cytochrome *c* rereduction decreased about 10-fold without significant changes in the amplitude (Figure 6). The difference in maximum amplitude of the WT and mutant traces reflect the cytochrome c_2 content, which can be highly variable from preparation to preparation.

DISCUSSION

Semiaerobic and Photosynthetic Growth. We noted that wild type and B187DN mutant cells grow photosynthetically at comparable rates, indicating that cytochrome bc_1 complex in mutant cells functions with rates similar to that of WT cells. In some experiments, we used semiaerobically grown cells. Despite the identical conditions for the semiaerobic growth of cells, we observed that the colors of WT and

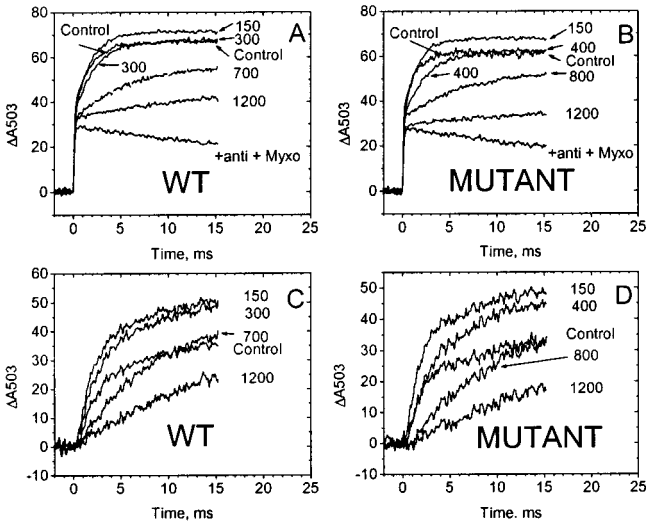

FIGURE 3: Effect of DCCD on the kinetics of phase III of the electrochromic carotenoid bandshift in WT (panels A and C) and mutant (panels B and D) chromatophores. Incubation medium: 50 mM MOPS, pH 7.5, and 100 mM KCl. 2 μM 1,2-naphthoquinone, 2 μM PMS were used as redox mediators, 1 mM Fe(III)EDTA was present as redox buffer. Concentration of bc_1 complex in chromatophores $\approx$ 0.25 μM. Panels A and B: Kinetics of the electrochromic carotenoid bandshift in WT and B187DN chromatophores, respectively, measured at different DCCD concentrations [indicated by numbers (μM) near curves]. Bottom trace is in the presence of antimycin A (10 μM) and myxothiazol (5 μM). Panel C and D: Phase III of electrochromic shift of carotenoids in WT and B187DN chromatophores, respectively, obtained by subtraction of the trace in the presence of antimycin and myxothiazol from the traces at different DCCD concentrations. Before subtraction the traces were normalized at 0.2 ms to minimize the differences in the amplitude of phases I and II of the electrochromic carotenoid bandshift.

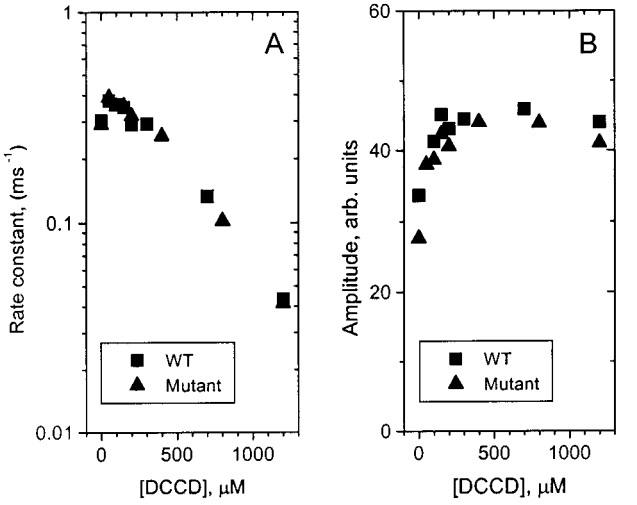

FIGURE 4: Dependence of the rate constant (A) and amplitude (B) of phase III of the electrochromic bandshift on DCCD concentration in WT (squares) and mutant (triangles) chromatophores, obtained from one-exponential fits of the kinetics shown in Figure 3.

mutant cell cultures in most cases were slightly different, indicating different compositions of the photosynthetic apparatus in WT and mutant cells. Thus, it appears that mutation of Asp^{B187} can influence the physiology of the cells, although this influence was not apparent under photosynthetic growth.

Stoichiometry of the Different Components in Mutant and WT Chromatophores. Knowledge of the stoichiometry

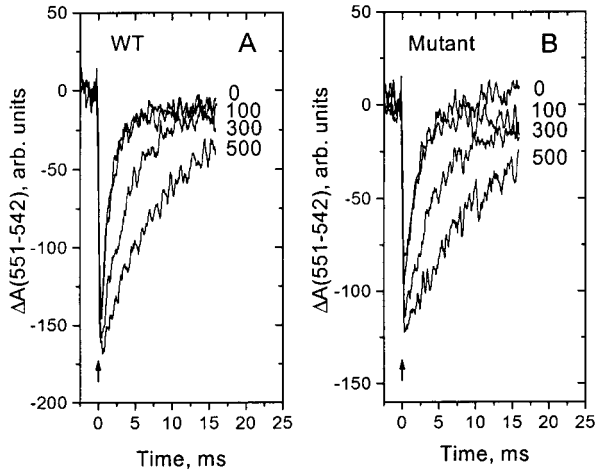

FIGURE 5: Effect of DCCD concentration (μM, indicated by numbers near curves) on the kinetics of cytochrome *c* rereduction in WT (A) and mutant (B) chromatophores. Incubation medium: 50 mM MOPS, pH 7.5, 100 mM KCl. 2 μM 1,2-naphthoquinone, 2 μM PMS were used as redox mediators, 1 mM Fe(III)EDTA was present as redox buffer. Concentration of bc_1 complex in chromatophores $\approx$ 0.25 μM. Curves were smoothed by 5-point averaging.

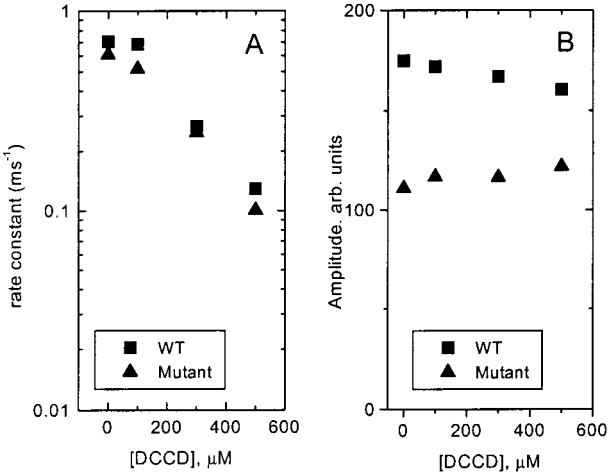

FIGURE 6: Dependence of the rate constant (A) and amplitude (B) of the kinetics of cytochrome (c_2 + c_1) rereduction on DCCD concentration in WT (squares) and mutant (triangles) chromatophores, obtained from one-exponential fits of the kinetics shown in Figure 5.

between different components of the electron transport chain is important for the quantitative analysis of WT and mutant B187DN chromatophores. In different chromatophore preparations, the ratio [RC]/[bc_1] was 1−3, both in WT and mutant. The average stoichiometry was indistinguishable from that reported for genomic wild type (*12*). Thus, the concentration of cytochrome bc_1 complexes was not significantly reduced in mutant chromatophores, even though the gene for the cytochrome bc_1 complex was located on the plasmid and not in the chromosome (see Experimental Procedures).

Properties of Wild Type and Mutant Chromatophores. In almost all respects, chromatophores from B187DN mutant cells are indistinguishable from WT chromatophores. In particular, the kinetics of cytochrome *b* reduction, as well as cytochrome *c* oxidation, are the same in both mutant and WT chromatophores (less than 15% differences in rates). We found negligible differences in both kinetics and amplitudes

of phase III of the electrochromic shift (less than 15% for the first two flashes when normalized per RC in chromatophores from photosynthetically grown cells) (Figures 2 and 4).

Effects Induced by DCCD. DCCD clearly has two different effects on the electrochromic shift of carotenoids. At low concentrations, DCCD increases the magnitude of the changes at 503 nm (see Figures 3 and 4). This is likely due to a decrease in the permeability of the membrane through F_0F_1 after DCCD binding to F_0, which is well-documented in the literature (*38*, *39*). At higher concentrations (>100 μM) DCCD inhibited the kinetics of phase III of the electrochromic shift (Figure 4) in both WT and B187DN mutant chromatophores. Our results agree well with the work of Takamiya (*40*) on WT *Rb. sphaeroides* chromatophores. Furthermore, this residue has little or no significance in the normal functioning of the cyt bc_1 complex, as evidenced by the following. First, replacement of aspartate 187 in cytochrome *b* by asparagine leads to not more than 15% inhibition of phase III of the electrochromic shift (when normalized per RC, Figures 1 and 4). Second, the kinetics of generation of electric potential in the bc_1 complex, judged from the antimycin-sensitive (Figure 2) and antimycin-insensitive myxothiazol-sensitive phases (Figure 2) of the electrochromic bandshift, were practically identical in WT and mutant chromatophores. Third, the kinetics of reduction of cytochromes *b* and *c* are the same in wild type and in B187DN mutant (Figures 1 and 5). Finally, DCCD has similar effects on phase III of the electrochromic shift in WT and in B187DN mutant (Figures 3 and 4).

The lack of inhibition by DCCD of the amplitude of phase III of the electrochromic shift demonstrates that the stoichiometry of charge movement through the bc_1 complex is unaffected. In the turnover of the bc_1 complex, electrogenicity is strictly coupled to proton uptake and release, regardless of the precise model. Thus, we conclude that there is no indication of decoupling in single flash-induced turnover of the bc_1 complex and that none of the effects of DCCD described here or earlier (*25*) can be explained by the binding of DCCD to aspartate 187 in cytochrome *b*. They may, however, be due to the cumulative effect of many modified amino acid residues. This is suggested by the observation of Cocco et al. (*19*) that the fluorescent hydrophobic nucleophile 4′-[(aminoacetamido) methyl]fluorescein (AMF), which requires a carboxyl residue modified by DCCD for formation of an amide bond, did not react with DCCD-treated bovine cytochrome *b*. The labeling of *Rb. sphaeroides* cytochrome *b* by ^{14}C-DCCD (*25*) may indicate that DCCD is bound to one or more nonacidic residues.

Another indirect indication of a cumulative effect of DCCD is the monoexponential character of the kinetics of the phase III at DCCD concentration > 100 μM (Figure 3). If a single amino acid were modified by DCCD, we should expect to observe two different kinetic components, corresponding to unmodified and modified bc_1 complexes, with relative amplitudes changing with DCCD concentration. However, at concentrations of DCCD >100 μM we were unable to separate two components of the phase III. It should be noted that in all the experiments described the membranes were washed to remove unbound DCCD. However, no significant difference was seen when the washing step was eliminated. The homogeneous kinetics cannot therefore be attributed to nonspecific effects of loosely bound DCCD.

We conclude, therefore, that Asp^{B187} plays no significant role in the electrogenic activity of bc_1 complex in chromatophores of *Rb. sphaeroides*. Furthermore, our results show that none of the observable effects of DCCD on cytochrome bc_1 complex function are due to modification of Asp^{B187}. Alternatives include modification of the quinone binding site or modification of docking of the iron−sulfur protein (*27*).

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