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The nature and magnitude of the charge-separation reactions of ubiquinol cytochrome c_2 oxidoreductase

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The transdielectric charge separation reaction catalyzed by the ubiquinol-cytochrome c_2 oxidoreductase is achieved in two fractional steps. We present a detailed analysis which addresses the nature of the charge transferred, the redox groups directly involved in charge separation and the contributions of each to the full charge separation catalyzed by the enzyme. Accounting for light saturation effects, reaction centers unconnected to cytochrome c_2 and the fraction of total cytochrome bc_1 turning over per flash permits detailed quantitation of: (1) the red carotenoid bandshift associated with electron transfer between ubiquinol at site Q_z and the high- (2Fe2S center, cytochrome c_1) and low-potential (cytochrome b_L, cytochrome b_H) components of cytochrome bc_1; (2) the blue bandshift accompanying reduction of cytochrome b_H by ubiquinol via site Q_c (the reverse of the physiological reaction); and (3) the effect of $\Delta\psi$ on the Q_c-cytochrome b_H redox equilibrium. Studies were performed at pH values above and below the redox-linked pK values of the redox centers known to be involved in each reaction at equilibrium. The conclusions of this study may be summarized as follows: (1) there is no transdielectric charge separation apparent in the redox reactions between Q_z and cytochrome b_L, 2Fe2S and cytochrome c_1 (in agreement with Glaser, E. and Crofts, A.R. (1984) Biochim. Biophys. Acta 766, 223–235), i.e., charge separation accompanies electron transfer between cytochrome b_L and cytochrome b_H; (2) the redox reactions between cytochrome b_L and cytochrome b_H and between cytochrome b_H and Q_c constitute the full electrogenic span; (3) electron transfer between cytochrome b_L and cytochrome b_H contributes approx. 60% of this span; (4) electron transfer between cytochrome b_H and Q_c contributes 45–55% as calculated from the blue bandshift or the $\Delta\psi$-dependent equilibrium shift; (5) there is no discernable pH dependence of the Q_z-cytochrome b_H or Q_c-cytochrome b_H charge-separation reactions; (6) cytochrome b_L, Q_z, 2Fe2S, and cytochrome c_1 are on

Abbreviations: ant, antimycin; myx, myxothiazol; cytochrome bc_1, ubiquinol-cytochrome *c* (or c_2) oxidoreductase; RC, photosynthetic reaction center; $[BChl]_2$, bacteriochlorophyll dimer of the photosynthetic reaction center; $[BChl]_2^+$, oxidized $[BChl]_2$ dimer; BPheo, bacteriopheophytin; Q_c, antimycin-sensitive, ubiquinone-mediated functional site of the ubiquinol-cytochrome c_2 oxidoreductase (also called Q_{in} or Q_i); Q_z, myxothiazol-sensitive, ubiquinone-mediated functional site of the ubiquinol-cytochrome c_2 oxidoreductase (also called Q_{out} or Q_o); Q_{pool}, the mobile, membrane-associated pool of ubiquinone-10; cytochrome b_L, the low-potential cytochrome *b*-566; cytochrome b_H, the high-potential cytochrome *b*-560; UHDBT, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide, Mops, 4-morpholinepropanesulfonic acid; Caps, 3-[cyclohexylamino]-1-propanesulfonic acid; PMS, *N*-methyldibenzopyrazine methylsulfate; PES, *N*-ethyldibenzopyrazine ethylsulfate; OHNQ, 2-hydroxy-1,4-naphthoquinone; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine.

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the periplasmic side out of the low dielectric part of the membrane while cytochrome b_H is buried in the low dielectric medium; (7) electron transfer is the predominant if not the sole contributor to charge separation; (8) Q_z and Q_c are on opposite sides of the membrane dielectric profile.

Introduction

The ubiquinol-cytochrome c_2 oxidoreductase (cytochrome bc_1) of *Rhodobacter sphaeroides* acts in concert with the photosynthetic reaction center protein (RC) to complete a cycle of electron transfer in anoxygenic photosynthesis (for reviews, see Refs. 1–3). The cyclic system is part of the chromatophore membrane of the bacterium. Electron transfer between RC and cytochrome bc_1 is mediated by a pool of ubiquinone (Q_{pool}) contained in the membrane and a complement of water-soluble cytochrome c_2 located in the periplasm. Following light activation the RC separates charge across the membrane to create a membrane potential ($\Delta\psi$) and to generate ubiquinol (QH_2) and ferricytochrome c_2. The cytochrome bc_1 complements the RC by utilizing the free-energy difference between QH_2 and ferricytochrome c_2 to catalyze further charge separation and proton gradient formation across the membrane. The electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) generated is used by the cell to drive phosphorylation and transport.

Of the two membrane proteins the RC is the best described, and in the present report it serves as a reference for the cytochrome bc_1. The charge separation that the RC catalyzes, directed across the membrane from the periplasm to the cytoplasm, has been shown to involve several distinct electron-transfer reactions, each step contributing to charge separation across a fraction of the membrane low dielectric. The existence of more than one contributing electrogenic charge transfer was first revealed [4] from resolved phases of electrochromic red shifts (called phases I and II) in the absorption bands of the $\Delta\psi$-reporting [5] carotenoid molecules contained in the membrane. These findings were later confirmed and extended by direct-voltage measurements [6–12] and, in certain reactions, by $\Delta\psi$-induced shifts in redox equilibria (see Refs. 11 and 13).

An important aspect regarding the use of the flash-activated carotenoid response to follow charge separation is that it is temperature insensitive and its use is unrestricted well into the submicrosecond range; this permits the kinetic details of electrogenic events beyond this time to be faithfully reported not only under ambient conditions [4,14,15], but also at cryogenic temperatures [16]. It is also pertinent to the current work that the flash-activated bandshifts have proven to be a useful exploratory tool with the RC to reveal not only individual steps of charge separation, but also, under suitably arranged conditions, intraprotein charge recombination which is reported as a blue shift [14].

In comparison, less is known about the details of charge separation reactions of the cytochrome bc_1. Although turnover of cytochrome bc_1 induces red bandshifts in the carotenoid spectrum of similar magnitude to the RC (termed Phase III; Refs. 4 and 17–19), only recently [20–22] has the question been investigated that there may be, in this type of redox protein, individual and fractional steps contributing to the transmembrane charge separation. This kind of investigation is feasible with the cytochrome bc_1 with current, detailed descriptions of the components and electron-transfer pathways. The present view of the cytochrome bc_1 electron transfer system is briefly summarized below.

(1) Electron transfer into and out of the *Rhodobacter* cytochrome bc_1 is governed by two protein-assocaited sites of quinone oxidation and reduction called Q_z and Q_c, respectively. Reduction of cytochrome *c* (or cytochrome c_2) occurs at a third site. Evidence that the postulated Q_z and Q_c sites [23,24] represent discrete structural entities has been provided by: (a) their mutually exclusive sensitivities to inhibitors [25]; (b) the existence of two types of cytochrome bc_1 mutant which each have lesions in the Q_z site while retaining wild-type activities assocaited with Q_c [26–28]; and (c) differences in their ability to stabilize the intermediate semiquinone; a stable semiquinone can be formed at equilibrium in the Q_c site [29].

(2) The first catalytic step in cytochrome bc_1 involves oxidation of QH_2 from the membrane-

bound Q_{pool} at the Q_z site [30–32]. One electron from Q_zH_2 passes to a high-potential sequence comprised of a tightly associated 2Fe2S center ($E_{m7} = 280$ mV; Refs. 33 and 34) and cytochrome c_1 ($E_{m7} = 280$ mV; Refs. 35 and 36) which then reduces the soluble cytochrome c_2 ($E_{m7} = 340$ mV; Refs. 35 and 36). Simultaneously (i.e., no stable semiquinone), a second electron from the QH_2 in the Q_z site reduces a low-potential sequence involving first the reduction of cytochrome b_L ($E_{m7} = -90$ mV; Refs. 37 and 38) and then cytochrome b_H ($E_{m7} = 50$ mV; Refs. 37 and 38). The reduced cytochrome b_H is then thought to reduce a Q that enters the Q_c site from the Q_{pool}, resulting in the formation of a semiquinone. This stable semiquinone is considered to remain in the Q_c site until, upon a second turnover of the cytochrome bc_1, it is reduced again to form the doubly reduced quinol which returns to Q_{pool} [3]. Thus two QH_2 molecules are required but only one is net oxidized per full turnover of the cytochrome bc_1.

(3) Early proposals for the function of the cytochrome bc_1 [23,24] suggested the locations for Q_z and cytochrome b_L along with 2Fe2S and cytochromes c_1 and c_2 on or near the periplasmic side of the membrane, and Q_c and cytochrome b_H on or near the cytoplasmic side. In this arrangement the charge separation (two unit charges per QH_2 oxidized) was proposed to occur between Q_z/cytochrome b_L and Q_c/cytochrome b_H and to involve electron transfer through the low dielectric medium of the membrane. However, recent studies have shown that a complete charge separation is achieved in at least two distinct steps involving the reduction of cytochrome b_H and its subsequent oxidation via site Q_c [20]. The questions that remain regarding these reactions concern the nature of the charge or charges moved in each electrogenic reaction and the distance they are translocated through the membrane dielectric medium.

The intradielectric positions of the Q_z and Q_c sites, as well as their positional relationships with cytochrome b_L and cytochrome b_H have direct bearing on the mechanism of charge separation [39]. Each of these redox centers [29,40] as well as the 2Fe2S [41] exhibits redox-linked protonations and deprotonations which accompany electron transfer at equilibrium. These protolytic reactions provide possibilities for electrogenic proton transfer. Depending on intradielectric positions of redox partners, a reaction may separate charge by the net movement of electrons or protons [42,43] or some distinct sequential combination of both through a fraction of the low dielectric medium. The transdielectric movement of a semiquinone anion has also been proposed as a possible charge-separation mechanism of cytochrome bc_1 (see for example Ref. 44).

In this study we have characterized a charge separation accompanying the reaction between cytochrome b_H and Q_c. Carotenoid bandshifts and the effects of $\Delta\psi$ on the reaction were rigorously calibrated with respect to the coupled redox events in order to provide more accurate quantitations of the electrogenic contribution of the cytochrome b_H-Q_c reaction. Preliminary, semiquantitative work on this reaction has been reported [45]; however, this was done without making several essential corrections to the measurements. Consequently, the electrogenic contribution to the overall charge separation was considerably underestimated. A similar level of quantitation has been applied to the Q_z-mediated reduction of cytochrome b_H permitting reevaluation of earlier estimates. In addition, we have addressed the issue of whether the electrogenic species in each of these reactions is an electron or proton or a combination of both. This has been done by examination of the charge separation over a pH range encompassing the redox-linked pK values of the redox cofactors.

Materials and Methods

Chromatophores were prepared from *Rb. sphaeroides*, strain Ga, as previously described [15]. Rapid kinetics were measured on a Johnson Foundation dual-wavelength spectrophotometer equipped to deliver short (full width at half height, 8 μs) flashes of actinic light [15]. Redox potentiometry was performed using the equipment and protocols described in Ref. 46. Buffers used for flash-induced kinetics experiments were as follows: 50 mM Mops (pH 7–7.5); 50 mM Tricine (pH 8–8.5); 50 mM glycine (pH 9–9.5); 50 mM Caps (pH 10–10.5). Each buffer was 100 mM in KCl. Further experimental details are provided in

each figure legend. Reaction center, cytochrome *b* and cytochrome *c* concentrations were determined using previously published extinction coefficients [15].

Antimycin, valinomycin, *N*-methyldibenzopyrazine methylsulfate (PMS), *N*-ethyldibenzopyrazine ethylsulfate (PES) and Good buffers were purchased from Sigma, myxothiazol from Boehringer-Mannheim and 2-hydroxy-1,4-naphthoquinone (OHNQ) and 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD) from Aldrich. All other chemicals were reagent grade and were purchased from commercial sources.

The following points outline the basis of the experiments described.

(1) Use of inhibitors; isolation of partial reactions in cytochrome bc_1

(a) Antimycin. Antimycin binds tightly and specifically to the Q_c site and blocks cytochrome b_H oxidation by Q_c [47] and cytochrome b_H reduction by Q_cH_2 [48,49]. In the presence of antimycin, cytochrome b_H reduction occurs via Q_z and cytochrome b_L. This reaction (i.e., 'oxidant-induced reduction') is driven by obligatory electron transfer through the 2Fe2S, cytochrome c_1 and c_2 [3,50,51].

Myxothiazol. Myxothiazol binds tightly and specifically at the Q_z site and blocks the simultaneous reduction by Q_zH_2 of 2Fe2S (and hence cytochrome c_1 and c_2) [52,53] and cytochrome b_L (and hence cytochrome b_H) [18,48,49,52,53]. However, it should be noted that in the presence of myxothiazol, electron transfer still can occur from 2Fe2S through the cytochrome c_1 and cytochrome c_2.

(b) Antimycin and myxothiazol. In the presence of both antimycin and myxothiazol, flash activated turnover of the cytochrome bc_1 is restricted to electron transfer between the 2Fe2S, cytochrome c_1 and cytochrome c_2.

(2) Flash-activated carotenoid bandshift measurements

(a) Experimental conditions. In all the flash-activated carotenoid bandshift measurements reported here, the reductant for cytochrome bc_1 was provided directly by the RC. Thus all experiments were performed with Q_{pool} poised oxidized prior to activation and hence, after the flash, QH_2 entered Q_{pool} and interacted with cytochrome bc_1 via the Q_z or Q_c sites. In each case, inhibited by antimycin or myxothiazol, respectively, cytochrome b_H reduction was observed.

(b) Standardizations for carotenoid bandshift measurements. The magnitude of the carotenoid bandshift associated uniquely with the cytochrome c_2-RC electrogenic reactions was used as a scale, against which the bandshifts associated with reactions of the cytochrome bc_1 are compared. At least two carotenoid bandshift phases have been resolved in the RC; Phase I associated with the oxidation of $[BChl]_2$ and reduction of Q_A and Phase II with the rereduction of $[BChl]_2^+$ by cytochrome c_2. Since one QH_2 is produced as substrate for the cytochrome bc_1 per two RCs, this means that two unit charges are moved from the cytochrome c_2 complement across the membrane through the RC per QH_2 produced. Hence, half the magnitude of bandshift associated with the production of one QH_2 represents the amplitude of bandshift generated by the movement of one charge across the entire membrane.

(c) Quantitation of cytochrome bc_1 carotenoid bandshift. In a normal, high-quality, coupled chromatophore preparation there are several factors that must be accounted for in quantitation of electrogenic events as reported by carotenoid bandshifts. Efforts were made to identify those electron-transfer events in the RC and cytochrome bc_1 that were directly coupled to carotenoid bandshifts and correct for those that were not. Also we corrected for reactions that for energetic or statistical reasons do not go to completion. These are summarized in Fig. 1 for a hypothetical experiment analyzing the cytochrome bc_1 bandshift after QH_2 oxidation at the Q_z site.

(i) Chromatophore sheets. In a normal preparation of chromatophores, 10–18% of the membranes are in the form of sheets. This fraction was assayed by measuring $[BChl]_2^+$ remaining oxidized 20 ms after a single flash in uncoupled chromatophores (valinomycin + nigericin) poised at an E_h of 100 mV. Any $[BChl]_2^+$ remaining at this point has no associated cytochrome c_2 inside the chromatophore. Addition of exogenous ferrocyt *c* (horse heart) causes complete $[BChl]_2^+$ reduction, confirming that the $[BChl]_2^+$ remaining was exposed to the external aqueous phase and was

reducible by the added cytochrome *c*. These sheets yield no carotenoid bandshift, as in Fig. 1a, and because there is no associated cytochrome c_2 do not elicit any reactions in cytochrome bc_1. Thus, this population was subtracted from the total $[BChl]_2$ in subsequent experiments.

(ii) Limitations on reduction of $[BChl]_2^+$ by cytochrome c_2. It has been shown that the electrogenic electron transfer from cytochrome c_2 to $[BChl]_2^+$ is impeded by $\Delta\psi$ [13,54]. It is also possible that statistical factors may control to some degree the collision and electron transfer between these centers. This has obvious effects on the amplitude of phase II of the bandshift. Hence, the contribution of phase II to the charge separation across the entire membrane (phase I + II), needed for the standardizations required above (subsection 2b. Standardizations for carotenoid bandshift measurements) can be underestimated. Thus experiments will yield a mixture of states b and c (Fig. 1). Corrections to obtain an amplitude that is representative of a completed reaction as shown in Fig. 1c were obtained as follows. The fraction of $[BChl]_2^+$ left oxidized 20 ms after a flash in the presence of inhibitor was calculated, accounting for flash saturation and for $[BChl]_2$ in sheets (flash saturation was measured at an E_h of 430 mV and was in the range of 89–92%). From these data a scaling factor (the 'lost cytochrome *c*' correction factor) was calculated and was used to multiply bandshift data to reflect a situation where all oxidized $[BChl]_2$ in closed, coupled vesicles was reduced by cytochrome c_2, i.e., as in Fig. 1c). The carotenoid bandshift measured in the presence of myx and ant was multiplied by the scaling factor to produce a complete phase I + II for every photoactivated RC donating a QH_2 to Q_{pool}. This corrected bandshift was used as the standar-

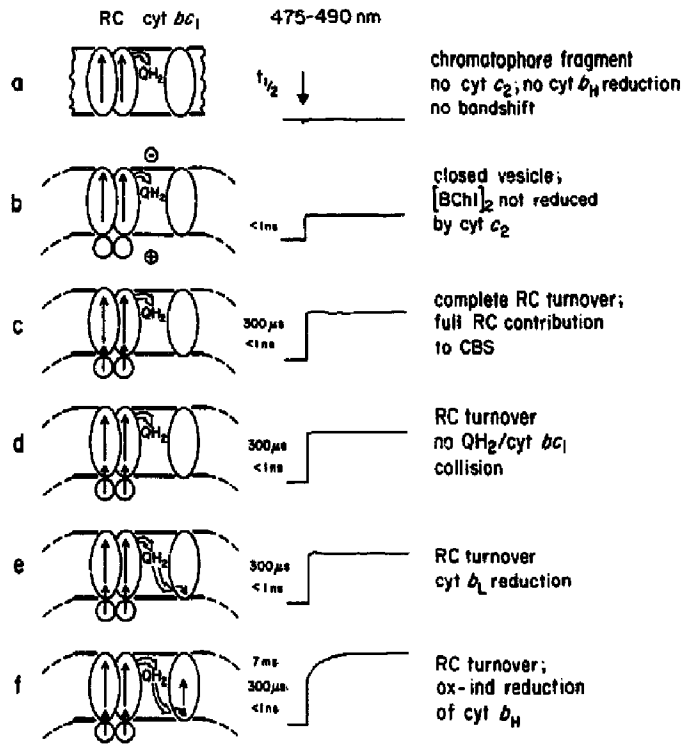

Fig. 1. Turnover of the cyclic electron-transfer system in various fractions of an antimycin-treated chromatophore preparation; contributions of each fraction to the carotenoid bandshift. These contributions were quantitated and used to correct bandshift data. The procedures for calibration are outlined in Materials and Methods. cyt, cytochrome.

dized absorbance change reflecting the movement of two charges across the full dielectric of the membrane.

(iii) Oxidation of QH_2 by cytochrome bc_1. The oxidation of QH_2 produced by RC appears to be controlled by statistical factors which relate the collisional frequency to turnover in cytochrome bc_1. These factors are particularly evident in experiments where Q_{pool} is poised oxidized prior to delivery of a flash. Thus, the maximum amplitude of cytochrome b_H reduction is only established after three or four flashes. Reasons for incomplete reduction after a single flash may be due to an absence of collision of QH_2 and cytochrome bc_1 (Fig. 1d) or a double collision of QH_2 with a cytochrome bc_1. (Fig. 1e). It is also possible that $\Delta\psi$ established by RC turnover may provide some energetic restriction on electron transfer between cytochrome b_L and cytochrome b_H. The amount of cytochrome b_L and cytochrome b_H reduced following the flash was determined using analysis of the spectral region between 555 and 575 nm (see Results). Ultimately, to provide a good basis for comparison from experiment to experiment, all data were corrected to reflect a perfect system (Fig. 1f), where each photoact results in complete $[BChl]_2$ reduction and QH_2 oxidation in a closed, coupled vesicle. Experiments which analyzed the Q_c-cytochrome b_H reaction were calibrated in an analogous manner.

Taking the above corrections into account, the extent of charge transfer in the cytochrome bc_1 occurring across part of the membrane dielectric profile (x) was determined using

$$x\,(\%) = \frac{\Delta A_{bc1}/m}{\Delta A_{RC}/2} \times 100$$

In this equation ΔA_{bc1} is the shift associated with events in cytochrome bc_1 and ΔA_{RC} is the corrected phase I + II bandshift. m is the number of charges moving through the membrane per cytochrome bc_1 in response to the Q_z-cytochrome b_H or Q_c-cytochrome b_H electron-transfer reactions.

(3) The effect of $\Delta\psi$ on the equilibrium of the Q_c-cytochrome b_H reaction

(a) Experimental conditions. Chromatophores were poised at pH 8 and at E_h 80 mV to establish the equilibrium constant for the reaction (see Eqn. 5) to be near unity. If the reaction is electrogenic, the equilibrium constant and hence the level of cytochrome b_H reduced after a flash should be maximally sensitive to the prevailing $\Delta\psi$.

(b) $\Delta\psi$ calibration. The $\Delta\psi$ generated on a per flash basis was calibrated by imposition of valinomycin-promoted potassium-diffusion potentials of known magnitude and simultaneous observation of carotenoid bandshift extent [5]. The magnitude of the bandshift developed after a single flash under experimental conditions used for observation of the Q_c-cytochrome b_H reaction was compared to the K^+ diffusion potential data to obtain $\Delta\psi$ generated by the RC.

(c) Quantitation. The amplitude of cytochrome b_H reduction was measured in the presence of myxothiazol and the presence and absence of valinomycin. In this way the $\Delta\psi$-induced shift in the equilibrium constant was determined. The analysis is similar to that used by Hinkle and Mitchell [55] and Takamiya and Dutton [13] and is described by;

$$x\,(\%) = \frac{E_h - E_h'}{m\,\Delta\psi} \times 100 \qquad (2)$$

In this equation E_h' and E_h are the redox potentials of cytochrome b_H, calculated from the Nernst equation, in the presence and absence of membrane potential, respectively ($\Delta\psi$ is the transmembrane potential, periplasmic minus cytoplasmic sides); m is the number of charges moved through the low dielectric medium of the membrane.

Results

The carotenoid bandshift generated by uninhibited cytochrome bc_1 when the Q_{pool} is oxidized

Fig. 2 shows the flash-induced carotenoid bandshift in uninhibited chromatophores in which the Q_{pool} is oxidized before activation. The data are shown for experiments performed at pH 7. The wavelength pair 475–490 nm was chosen to observe the bandshift. After an initial first phase (containing the unresolved phases I and II associated with the RC) there is a slow phase that reaches a maximum after about 30 ms. This slow phase is abolished by addition of antimycin and

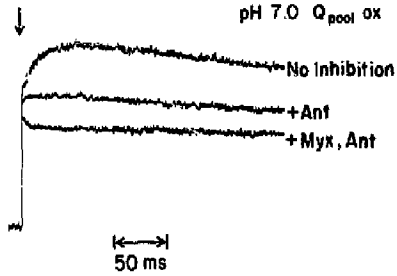

Fig. 2. Carotenoid bandshift kinetics in chromatophores of *Rhodobacter sphaeroides* Ga at pH 7. Chromatophores were suspended in 50 mM Mops, 100 mM KCl (pH 7) to a final RC concentration of 0.2 μM. 5 μM OHNQ, 2.5 μM DAD, and 1 μM each of PMS, PES and pyocyanine were added as redox mediators. The ambient E_h was adjusted to poise the Q_{pool} oxidized, i.e., approx. 220 mV. A single flash was delivered and the kinetics of the optical change at the wavelength pair 475–490 nm was observed. Where indicated, antimycin was added to 5 μM and myxothiazol to 3 μM.

myxothiazol, consistent with it arising from charge separation in cytochrome bc_1. The amplitude of the slow phase is about half of that which is generated optimally with QH_2 already present in the Q_{pool} (not shown); this result is consistent with earlier findings [3,20].

The lack of carotenoid bandshift generated by electron transfer through the high-potential 2Fe2S, cytochrome c_1 and c_2 segment

After the addition of myxothiazol and antimycin (Fig. 2), some contributions to the remaining bandshift could conceivably arise if charge separation was associated with electron transfer from 2Fe2S to cytochrome c_1 ($t_{1/2}$ = 200 μs; [56,57]) and from cytochrome c_1 to cytochrome c_2 ($t_{1/2}$ = 150 μs [57]). This possibility was pursued by exhaustive examination of the bandshift under a wide range of conditions of pH (from pH 7 to 10 and redox potential (E_h = 80–400 mV). The bandshifts were measured with different components of the 2Fe2S and cytochrome c_1/c_2 trio oxidized or reduced before activation. Further examination was also made when electron transfer between 2Fe2S and cytochrome c_1 was eliminated by use of UHDBT instead of myxothiazol [34]. The results (not shown) failed to reveal any evidence for charge separation in this segment of the electron-transfer system. This evidence supports the interpretation that; (a) there is no electrogenic reaction between 2Fe2S, cytochrome c_1 and cytochrome c_2; and (b) antimycin and myxothiazol together abolish all electrogenic reactions in the cytochrome bc_1.

The carotenoid bandshift associated with the photosynthetic reaction center

A corollary of the final conclusion in the previous section is that, in the presence of antimycin and myxothiazol, the fast bandshift phase is due to electron transfer associated with the cytochrome c_2-RC and represents the net transfer of two charges across the dielectric per flash, i.e., per QH_2 formed; similar conclusions were reported in Ref. 20. This amplitude is used to provide a value for ΔA_{RC} for Eqn. 1 subject to the corrections outlined in Materials and Methods.

Q_z mediated cytochrome b_H reduction and the carotenoid bandshift

In the presence of antimycin, oxidant-induced reduction of cytochrome b_H occurs with a $t_{1/2}$ of approx. 7 ms when Q_{pool} is poised oxidized before flash activation [2]. In Fig. 2 the difference between the trace generated in the presence of antimycin minus that generated by the RCs is evidence for an electrogenic charge separation in the mechanism of electron transfer from the Q_z site through cytochrome b_L to cytochrome b_H. These data are in agreement with Ref. 20. The contribution, x, of this charge movement to the total bandshift may be obtained after following carefully the correction protocols as outlined in Materials and Methods and below.

Fig. 3 shows spectra in the region between 530 and 585 nm taken 30 ms after one or two flashes delivered at pH 7. The spectra in the presence of antimycin (panel B) were compared to one obtained with antimycin plus myxothiazol (panel A). Note that more cytochrome c oxidation occurs in the presence of myxothiazol due to the block of electron transfer between Q_z and 2Fe2S (see also Ref. 26). The difference in the region between 560 and 570 nm is due to the two b-type cytochromes (cytochrome b_L ΔA_{max} = 566 nm; cytochrome b_H ΔA_{max} = 560 nm [58]) and clearly shows contributions from each. The contribution of cytochrome b_L at 566 nm is particularly evident on the second

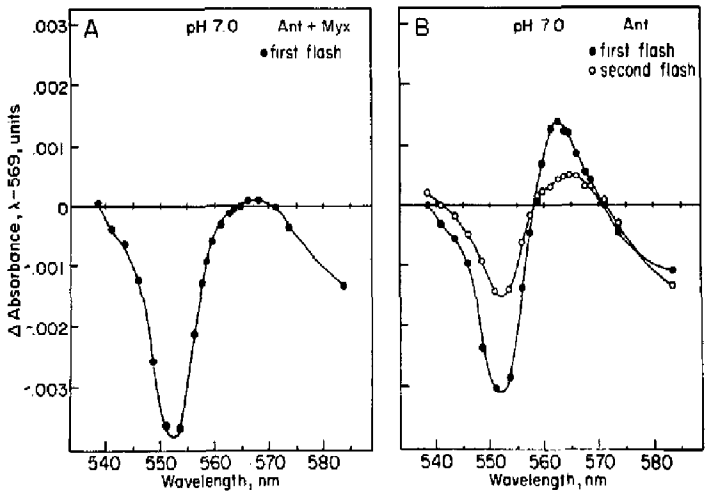

Fig. 3. Spectra of flash-induced optical changes in the 535–585 nm region during oxidant-induced reduction. Experimental conditions were as described in Fig. 2. The measuring wavelength was varied while a constant reference wavelength of 569 nm was maintained. The optical change was recorded 30 ms after flash delivery. The spectrum in panel A was obtained after addition of antimycin and myxothiazol.

TABLE I

CHARGE-SEPARATION MAGNITUDE OF THE Q_z-CYTOCHROME b_H REACTION VS. pH

The extent of the carotenoid bandshift due to electron transfer in cytochrome bc_1 was measured and compared to the corrected bandshift elicited by photoactivated turnover of the RC. Light saturation of RC was measured at an E_h of 430 mV. The proportion of RC in sheets, i.e., those whose turnover does not generate $\Delta\psi$ or contribute to the bandshift, was determined by measuring the difference in the number of RC reduced 20 ms after a flash in the presence and absence of exogenously added horse-heart cytochrome *c*. This is determined in the presence of valinomycin (5 μM) and nigericin (2 μM), i.e., in an uncoupled system. The proportion of RC in sheets was subtracted at the outset of experiments. This population, between 10 and 18% of the total RC in a typical preparation, does not exhibit oxidant-induced reduction (see Materials and Methods). The bandshift magnitudes are expressed in arbitrary units derived from calibrated absorbance differences.

pH	Phases I and II [a]; measured	Lost cytochrome *c* correction factor [b]	Phases I and II; corrected [c]	Bandshift; (+ant)−(+ant,myx)	Cytochrome b_H factor [d]	% full charge separation
7.0	37.0	1.166	43.14	11.0	1.14	56.9 (±2.1) [f]
7.5	35.5	1.139	40.40	8.8	1.18	51.2
8.0	35.0	1.250	43.75	11.5	1.21	63.4 (±0.8) [f]
8.5	33.0	1.104	36.40	9.8	1.18 [e]	63.4
9.0	32.0	1.244	39.80	9.4	1.18 [e]	55.8
9.5	31.1	1.076	33.40	9.3	1.19	65.8 (±3.6) [f]
10.0	30.9	1.130	35.00	10.1	1.18 [e]	67.9
10.5	28.0	1.074	30.10	6.9	1.18 [e]	53.6

[a] Phases I+II were measured in the presence of antimycin (3 μM) and myxothiazol (3 μM) with Q_{pool} poised oxidized.

[b] The lost *c* correction factor is derived from the proportion of $[BChl]_2^+$ in vesicles which are not reduced within 20 ms following a flash delivered in the absence of valinomycin.

[c] The corrected phases I+II number is derived from the product of a and b and represents the bandshift elicited when two charges pass across the full membrane dielectric.

[d] The cytochrome b_H factor corrects the number of cytochrome b_H reduced if each QH_2 produced by RC reduces cytochrome b_H in a cytochrome bc_1 complex.

[e] Cytochrome b_H factors were determined at pH 7.0, 8.0 and 9.5 and averaged to provide a factor (1.18±0.04) for use at other pH values.

[f] Standard deviation values are the cumulative sum of deviations from multiple determinations of the lost *c* and cytochrome b_H correction factors and the measured values of phases I+II and the fraction of full charge separation.

flash [38]. It is also clear, however, that more cytochrome b_H is reduced on the second flash, i.e., all cytochrome b_H is not reduced on the first flash with Q_{pool} oxidized. This point is important to the quantitation of the carotenoid bandshift amplitude accompanying this reaction.

These spectra were deconvoluted after assessment of the additive individual contributions of the cytochrome b_L and cytochrome b_H spectra. The method involved a simple fitting of pure spectra of the two cytochromes to the total difference spectrum. This method yields an approximate total cytochrome b_L or cytochrome b_H reduced after multiple flashes, or the fractional amounts of total cytochrome b_L or cytochrome b_H reduced after single flashes. The number of cytochrome b_H reduced as a function of the number of QH_2 generated by the RC was expressed as a factor (see Table I) by which the bandshift was multiplied to correct for cytochrome bc_1 complexes which do not receive a QH_2 or those in which QH_2 has reduced cytochrome b_L 30 ms after the flash.

Taken together with the corrections to the cytochrome c_2-RC derived bandshift, the fractional contribution for the charge transfer during oxidant-induced reduction was calculated. The details of the correction procedure are explained in the legend to Table I which shows data for the bandshift at pH values from 7.0 to 10.5. This range of ambient pH values was chosen to cover the pK values for the cytochrome b_H [59] and 2Fe2S center [41] in an attempt to study any pH dependence of the bandshift magnitude. The data, however, show no apparent trend as pH is increased. The values for the percentage of the total charge separation vary around a mean of 59.8% (+/− 6.1%).

Q_c-mediated cytochrome b_H reduction and the carotenoid bandshift

The remaining 40% of the carotenoid bandshift can be presumed to accompany events following the oxidation of cytochrome b_H via Q_c. The approach to the problem of directly measuring carotenoid bandshift amplitudes and/or of locating Q_c within the electrogenic span is complicated by the complex redox chemistry of Q_c. Direct measurement after initiation of the reactions via the Q_z site is obscured by the difficulty of isolating individual, single-electron redox steps involved in reducing Q_c first to the semiquinone and then to the quinol [29]. However, the question of the electrogenic character of the reaction may be addressed more directly by studying the cytochrome b_H-Q_c interaction in a direction opposite to that observed under physiological conditions. Cytochrome b_H reduction via Q_c has been shown to be feasible thermodynamically [29] and kinetically at high pH [48,49]. Hence, this approach avoids the complications of activation via the Q_z site.

The Q_c-mediated route to cytochrome b_H reduction is in essence a reversal of the physiological, second, single-electron reduction of Q_c by cytochrome b_H; that is, the reduction of semiquinone to quinol which is followed by QH_2 release into Q_{pool}. Thus, in the reverse of this reaction, quinol from Q_{pool} binds to the Q_c site (reaction 3 below) and reduces cytochrome b_H (reaction 4 or 5, depending on the pH), presumably leaving a semiquinone in the Q_c site. At pH values greater than the Q_c site-associated pK at 9 (for *Rhodobacter sphaeroides* [29]), the Q_c equilibrium redox properties indicate that the electron-transfer from cytochrome b_H is coupled to the release of one proton from QH_2 while another H^+ (in parenthesis) remains associated with the Q_c site (Eqn. 4). At pH values lower than the pK, the reaction is considered to be as shown in Eqn 5 which indicates the release of two protons.

$$QH_{2\,pool} + Q_c\ site = Q_cH_2 \qquad (3)$$

$$Q_cH_2 + \text{ferricytochrome } b_H = (H^+)Q_c^- + \text{ferrocytochrome } b_H + H^+ \qquad (4)$$

$$(H^+)Q_cH_2 + \text{ferricytochrome } b_H = (H^+)Q_c^- + \text{ferrocytochrome } b_H + 2H^+ \qquad (5)$$

Occurrence of a blue carotenoid bandshift with cytochrome b_H reduction. In the presence of myxothiazol at pH values greater than 9.0 and E_h values where Q_{pool} is oxidized, cytochrome b_H reduction via the Q_c route is favored. Under conditions of flash-activated multiple turnover the total amount of cytochrome b_H reduced after four flashes, however, is less than that observed for oxidant-induced reduction via Q_z (not shown).

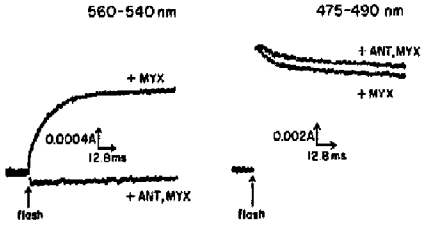

Fig. 4. Kinetics of cytochrome b_H reduction via Q_c and the associated carotenoid bandshift. Chromatophores were suspended in 50 mM glycine (pH 9.5), 100 mM KCl to a final RC concentration of 0.2 μM. Myxothiazol (5 μM) and antimycin (5 μM) were added where indicated. The cuvette contained the following redox mediators: PMS, PES, pyocyanine (1 μM); DAD (2.5 μM) and OHNQ (3 μM). The ambient redox potential was adjusted to maintain Q_{pool} oxidized before the flash. Turnover was initiated by delivery of a short (8 μs) pulse of actinic light. Cytochrome *b* was observed at the wavelength pair 560–540 nm and the carotenoid bandshift at 475–490 nm. A, absorbance.

This is expected because of limitations placed on the reduction of $[BChl]_2$ between flashes when Q_z is blocked by myxothiazol rather than on a lower amount of cytochrome b_H available for reduction via Q_c (Robertson, D.E. and Dutton, P.L., unpublished results).

A typical flash induced cytochrome b_H reduction trace is shown on the left of Fig. 4; the reaction is complete in approx. 30 ms and is inhibited by antimycin. On the right of Fig. 4 are kinetic traces showing the change in the carotenoid bandshift measured at 475–490 nm under identical conditions. In the presence of myxothiazol alone the amplitude of the red carotenoid bandshift is smaller than that observed with both inhibitors present (see also Ref. 60). The difference is considered to be due to a blue shift in the carotenoid absorption bands due to charge recombination associated with the reduction of cytochrome b_H via the Q_c site.

Kinetic matching of flash-activated cytochrome b reduction and the blue carotenoid bandshift. If the blue shift is coupled to cytochrome b_H reduction, the kinetics of these two phenomena should be identical. Fig. 5 shows a trace of Q_c-mediated cytochrome b_H reduction together with a subtracted trace of the time-dependent change in the carotenoid spectrum occuring under the same conditions. These two phenomena display similar kinetic behavior.

Redox-potential dependence of the amplitude of flash-activated cytochrome b_H reduction and the blue carotenoid bandshift. If the blue bandshift is a manifestation of transmembrane charge transfer linked to cytochrome b_H reduction, the redox potential dependence of the two phenomena should be strictly correlated. The top panel of Fig. 6 shows redox titrations of the carotenoid bandshift associated with the RC alone and when the Q_c-mediated cytochrome b_H reduction occurs. The difference between the two conditions, the blue carotenoid bandshift, is shown in the center panel where it is compared with the amplitude of flash-induced cytochrome b_H reduction.

The appearance of flash-induced cytochrome b_H reduction over the 10–90 mV E_h range is consistent with known $E_{m9.5}$ of 20 mV of cytochrome b_H [37,38]. However, cytochrome b_H reduction via the Q_c route is also affected by the state of reduction of the Q_{pool} ($E_{m9.5} = -60$ mV) and this leads to a complicated Nernst curve (see Ref. 49). At higher E_h values the amplitude of cytochrome b_H reduction also diminishes; this is due to complications with the cytochrome c_2-RC performance at these high E_h values (Robertson, D.E. and Dutton, P.L., unpublished results). At all values of E_h from 0 to 275 mV the ratios of the magnitudes of cytochrome b_H reduction and the

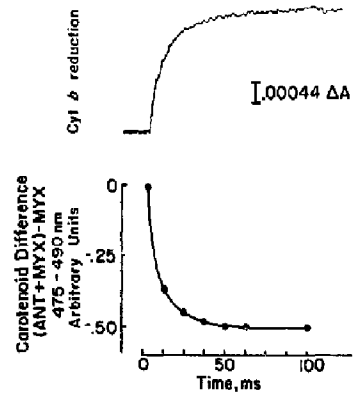

Fig. 5. Kinetics of antimycin-sensitive cytochrome b_H reduction and of the formation of the carotenoid blue shift. Experiments were performed as outlined in Fig. 4. The carotenoid difference was calculated at time points chosen from traces obtained in the presence of myxothiazol and myxothiazol plus antimycin. A, absorbance.

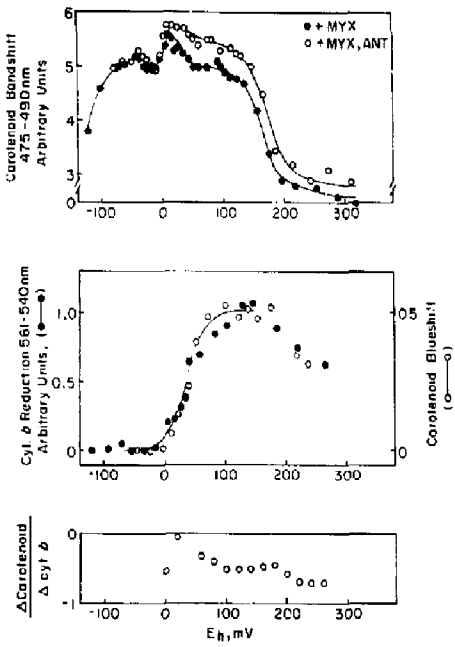

Fig. 6. Redox potential dependence of the carotenoid bandshift accompanying antimycin-sensitive cytochrome b_H reduction. Experimental conditions were identical to those in Fig. 4. The ambient redox potential was adjusted by incremental additions from freshly prepared solutions of sodium dithionite or potassium ferricyanide.

carotenoid difference are, within a reasonable experimental uncertainty, the same (lower panel). Thus, there is a good correspondence between the E_h dependence of the magnitudes both of cytochrome b_H reduction and the blue shift of the carotenoids.

Fig. 7 shows spectra of optical changes 30 ms after flash initiation of turnover in the presence of antimycin and myxothiazol (panel A) or myxothiazol alone (panel B). The spectra clearly show the reduction of cytochrome b_H via Q_c in the 560 nm region of panel B; there was no indication of cytochrome b_L reduction even after multiple flashes (not shown). As was done for the Q_z-cytochrome b_H reaction, the fraction of the total cytochrome b_H reduced per QH_2 provided by the RC was determined and then corrected for the fraction of cytochrome b_H unreduced (the cytochrome b_H correction factor). The total reducible cytochrome b_H is the amount observed via the Q_z-cytochrome b_H reaction at pH 9.5. The cytochrome b_H correction factor was used in conjunction with the cytochrome c_2-RC bandshift correction procedure to arrive at the percentage contributions tabulated in Table II for three pH values. Note that the lesser amount of cytochrome b_H reduced per flash via Q_c than via Q_z at an equivalent pH is reflected in a larger cytochrome b_H correction factor. In the case of pH 9.5 approximately half the available cytochrome b_H is

TABLE II

CHARGE-SEPARATION MAGNITUDE OF THE Q_c-CYTOCHROME b_H REACTION

The procedures for derivation of the bandshift proportion of this reaction are detailed in the legend of Table I.

pH	Phases I and II; measured [a]	lost cytochrome c_2 correction factor [b]	Phases I and II; corrected [c]	bandshift; (+myx)-(+ant,myx)	Cytochrome b_H factor [d]	% full charge separation
9.5	31.9	1.076	34.2	3.5	2.28	45.6 (±2.3) [e]
10.0	33.5	1.120	37.5	3.6	2.41	45.0 (±1.3) [e]
10.5	31.0	1.056	32.7	4.0	1.92	51.3 (±6.1) [e]

[a] Phases I+II were measured in the presence of antimycin (3 μM) and myxothiazol (3 μM) with Q_{pool} poised oxidized.

[b] The lost c correction factor is derived from the proportion of $[BChl]_2^+$ in vesicles which are not reduced within 20 ms following a flash delivered in the absence of valinomycin.

[c] The corrected phases I+II number is derived from the product of a and b and represents the bandshift elicited when two charges pass across the full membrane dielectric.

[d] The cytochrome b_H factor corrects the number of cytochrome b_H reduced if each QH_2 produced by RC reduces cytochrome b_H in a cytochrome bc_1 complex.

[e] Standard deviation values are the cumulative sum of deviations from multiple determinations of the lost c and cytochrome b_H correction factors and the measured values of phases I+II and the fraction of full charge separation.

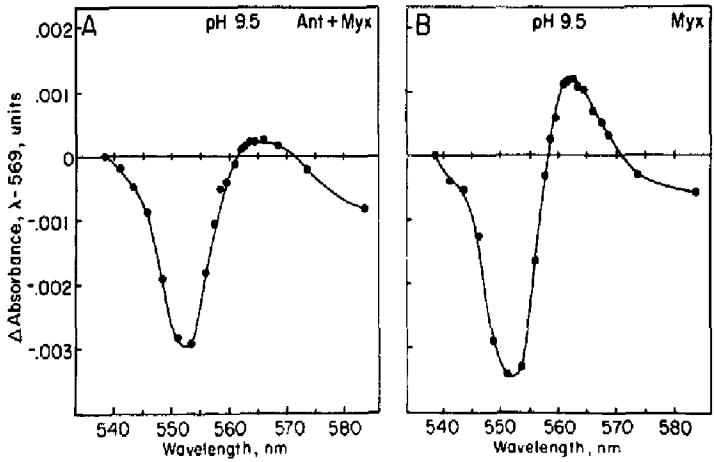

Fig. 7. Spectrum of optical changes accompanying cytochrome b_H reduction via Q_c. Conditions were as described in Fig. 4. Spectra were constructed as in Fig. 3. The spectrum in panel A was obtained in the presence of antimycin and myxothiazol to prevent cytochrome *b* reduction.

reduced via Q_c than is observed via the Q_z site (data not shown).

The pH range between 9.5 and 10.5 is the area in which the blue shift is easily visualized and also the range in which the reaction between Q_c and cytochrome b_H is described by reaction 4. There was no apparent pH dependence observed in the amplitudes of the blue bandshifts. The mean value (from three determinations) for the percent of the total normalized carotenoid bandshift that occurs with the redox reaction between Q_c and cytochrome b_H was 47.3 ($\pm$3.5%).

Thus, it can be concluded from the bandshift data that some mechanistic aspect of the Q_c-cytochrome b_H electron-transfer reaction is electrogenic at pH values above the pK of the Q_c site. Depending on the relative position of Q_c with respect to cytochrome b_H in the membrane profile, Eqn. 4 indicates that the source of the blue shift could be: (a) electron movement from Q_c to cytochrome b_H in a direction from the cytoplasmic side towards the periplasmic side; or (b) a proton from QH_2 moving in the opposite direction as a response to a non-electrogenic electron transfer from Q_c to cytochrome b_H, i.e., Q_c and cytochrome b_H are at the same position in the low dielectric profile of the membrane; or (c) partial contributions from (a) and (b). These possibilities might conceivably be explored by observing the bandshift magnitude above and below the pK of Q_c. Presumably, if one H^+ leaves the site above the pK, i.e., $m = 1$ (Eqn. 4) and two H^+ below the pK, $m = 2$ (Eqn. 5), the bandshift amplitude per cytochrome b_H reduced should double as the pH is lowered if protons are the electrogenic species. Unfortunately, the reaction is less favorable at lower pH. An experimental strategem is available, however, to resolve these possibilities by studying the effect of imposed field, i.e., $\Delta\psi$, on the amount of cytochrome b_H reduced at a pH below the pK of Q_c (reaction 5).

Effect of electric field on the redox equilibrium between Q_c and cytochrome b_H

At pH $\approx$ 8, the equilibrium constant of the Q_c-cytochrome b_H is close to unity and thus is optimally sensitive to test the affect of $\Delta\psi$. Single turnovers of the RC were used to establish $\Delta\psi$ prior to QH_2 oxidation by cytochrome bc_1. The effect of this $\Delta\psi$ on reduction levels of cytochrome b_H was studied by constructing spectra in the 530–585 nm region following single flash activation in the presence and absence of valinomycin.

Spectra were first obtained plus and minus valinomycin in the absence of any cytochrome *b*

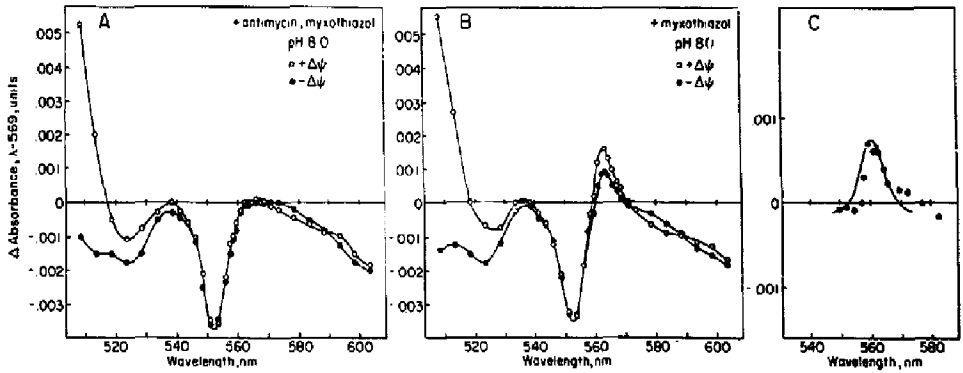

Fig. 8. Spectral resolution of the membrane potential effect on cytochrome b_H reduction. Experiments were performed in 50 mM Mops 100 mM KCl (pH 7.0) using 569 nm as a reference wavelength. Kinetics traces were accumulated at each wavelength minus the 569 nm reference and the extent was plotted at 25 ms following delivery of a flash. The data in panel A were obtained in the presence of saturating amounts of myxothiazol and antimycin to prevent cytochrome b_H reduction by either the Q_z or Q_c routes. Only optical changes due to cytochrome $c_1 + c_2$ and RC oxidation-reduction and to the carotenoid bandshift are observed. Valinomycin was subsequently added to 5 μM to cancel any optical contribution from carotenoid. A similar stratagem was applied in panel B with the exception that antimycin-sensitive cytochrome b_H reduction was allowed to proceed, i.e., no antimycin. Again, spectra were obtained in the absence and presence of valinomycin to isolate the carotenoid spectral contribution. Panel C is a difference spectrum which isolates the spectral changes due to $\Delta\psi$ during antimycin-sensitive cytochrome b_H reduction.

reduction (+antimycin and myxothiazol; Fig. 8A). There are clearly contributions from carotenoid as well as from cytochrome c_1 and cytochrome c_2 oxidation in the spectrum taken without valinomycin. Subtracting the optical changes observed in the presence of valinomycin leaves a spectrum due to carotenoid under conditions similar to those used for subsequent experiments.

Fig. 8B shows that, in the absence of antimycin, reduction of cytochrome b_H occurs. In the presence of $\Delta\psi$ (positive on the periplasmic side relative to the cytoplasmic side of the membrane) there is a further increase in the optical change after the flash in the 560 nm region. If the $\Delta\psi$-dependent difference between the spectra of panel A (i.e., the spectrum of carotenoid) is subtracted from the $\Delta\psi$-dependent difference between the spectra of panel B, the resulting spectrum should be an indication of the amount of $\Delta\psi$-dependent cytochrome b_H reduction. This procedure shows that the $\Delta\psi$ generated on a single flash increases the 561–569 nm absorbance significantly (panel C). In three experiments the increased level of reduction after a flash was between 0.026 and 0.035 μM, or approx. 25% of the total cytochrome b_H available.

Eqn. 2 was used with these data to estimate the extent of the electrogenic contribution of the Q_c-cytochrome b_H reaction. The value for $\Delta\psi$ for the first flash generated under identical conditions was between 45 and 55 mV (three determinations). Assuming a single charge is involved, the percentage contribution of this reaction (the x-value) is between 42 and 55. The mean value (from three determinations) for the contribution of this reaction is 49%, in close agreement with that calculated from the calibrated bandshift at pH 9.5. The suggestion from these two sets of data is that electrons and not protons move through the low dielectric in this reaction and that Q_c is on the outside of the membrane.

Discussion

The data summarized in Results provide experimental support for the idea that transdielectric charge separation events accompany two redox reactions in the cytochrome bc_1. These reactions were isolated experimentally and were demonstrated independently of each other. Both directed electrons to the same component, i.e., cytochrome b_H was reduced either via the Q_z site or via the Q_c

site. In the latter case (see Eqns. 3–5) the reduction reaction is occurring in a direction which is net opposite to that operating physiologically. This represents a reversal of a reaction that is considered to occur in the cytochrome bc_1 on every second oxidation reaction of cytochrome b_H. The physiological reaction results in the reduction of the semiquinone in Q_c (Q_c^-) to the quinol.

It is essential to note that, after a saturating flash delivered in the presence of either antimycin or myxothiazol, not all cytochrome b_H is reduced. It is clear from the data of Figs. 3 and 7 that after a first flash some cytochrome bc_1 does not receive reducing equivalents. A reasonable interpretation of data indicating incomplete turnover of the cytochrome bc_1 population is that statistical factors predominate in determining effective collisions between QH_2 from Q_{pool} and either of the two functional sites of the cytochrome bc_1 complex. If some cytochrome bc_1 encounter one QH_2, some two and some none, the distribution of cytochrome bc_1 turning over on any given flash could be represented by a Poisson distribution. Consistent with this view is the well-known observation that, with uninhibited cytochrome bc_1, the carotenoid bandshift magnitude is smaller by approximately one-half when Q_{pool} is oxidized (a typical trace under these conditions is shown in Fig. 2) as compared to the situation when QH_2 is in excess (Q_{pool} reduced). This possibility is currently under investigation (Robertson, D.E., Moser, C.C. and Dutton, P.L., unpublished results). Whatever the exact reason for heterogeneity, the important point for the present work was that all forms of heterogeneity were corrected for in quantitation.

The nature and magnitude of the charge-separation reactions

Our interpretation of the corrected data is that electron transfer through the low dielectric medium between cytochrome b_L and cytochrome b_H and between cytochrome b_H and Q_c is responsible for practically all of cytochrome bc_1 charge separation. In fact, the contributions of the cytochrome b_L to cytochrome b_H and the Q_c to cytochrome b_H reactions account for the entire membrane dielectric distance (in the range of $107 \pm 9\%$). Experiments which isolated the Q_z-2Fe2S-cytochrome c_1-cytochrome c_2, 2Fe2S-cytochrome c_1-cytochrome c_2, or cytochrome c_1-cytochrome c_2 sequences failed to show any contribution of these redox reactions to the cytochrome bc_1 charge separation.

The evidence for cytochrome b_L-cytochrome b_H charge separation during the Q_z-mediated oxidant-induced reduction is based in part upon the interpretation of Glaser and Crofts [20] who isolated the Q_z-cytochrome b_L reaction by careful redox poising. Their data supported the idea that electron transfer between the Q_z and cytochrome b_L produced no carotenoid bandshift at pH 8.35. The conclusion that electron transfer between the *b*-cytochromes was responsible for part of the electrogenic reaction of cytochrome bc_1 is substantiated by the work of Gopher and Gutman [61] who studied the effect of $\Delta\psi$ on the cytochrome b_L-cytochrome b_H equilibrium in mitochondrial cytochrome bc_1.

Our concerns have focused on the quantitation of the carotenoid bandshift elicited by the reaction between cytochrome b_L and cytochrome b_H. Glaser and Crofts [20] reported that with Q_{pool} oxidized prior to activation, the contribution was 35%, and with Q_{pool} reduced it was 50% of the full cytochrome bc_1-dependent bandshift [20]. It can be appreciated from the results presented here that the difference between these two numbers is related to the availability of QH_2 in the prepoised Q_{pool}, the fraction of the total cytochrome b_H subsequently reduced after a single flash and the number and extent of corrections required to standardize the responses using uncorrected data. Our own work with Q_{pool} oxidized is in good agreement with the work by Glaser and Crofts. Applying corrections, particularly the cytochrome b_H factor, to data obtained with Q_{pool} oxidized adjusts this value to approx. 60%. We chose to work with the Q_{pool} oxidized so that both the Q_c- and Q_z-mediated routes to cytochrome b_H reduction could be examined under the same starting conditions (the Q_c route is blocked with Q_{pool} reduced [49]). We have not examined the Q_z route with the Q_{pool} reduced; however, it is well-known that, compared to the situation when Q_{pool} is oxidized prior to activation, more cytochrome b_H is reduced after a flash. Hence the 50% value obtained [20] with Q_{pool} reduced is expected to be

closer to the fully corrected 60% value.

The bandshift due to Q_z-mediated cytochrome b_H reduction was quantitated over a range of pH values which encompassed the known pK values of cytochrome bc_1 redox-linked acid-base groups. This study was motivated by the idea that observation of the reaction at one pH value might miss a proton movement contributing to the bandshift (above the pK of a functional redox group) or that proton movement might cancel the electrochromic effect of electron transfer (below the pK of a functional group). In fact, our data reveal no change in electrochromism due to redox-linked protonation/deprotonation reactions when oxidant-induced reduction operates between pH 7.0 and pH 10.5. The data are, then, consistent with the interpretation that an electron transfer between the two *b*-cytochromes is responsible for the charge separation event following oxidation of QH_2 at Q_z.

Our data also provide no evidence for proton involvement in the reaction between Q_c and cytochrome b_H. No significant difference was seen in the charge separation when the reaction between QH_2 and cytochrome b_H was observed below and above the pK of the Q_c site, evidence for an electron as the charged group moving through the dielectric. The fact that the value for charge separation magnitude sums with that for Q_z-cytochrome b_H to approx. 100% also supports the idea that the charge separation mechanism is the same for electron transfer from cytochrome b_H to either Q or semiquinone in the Q_c site. In other words, electrons are the electrogenic species in the oxidation of cytochrome b_H via Q_c during physiological turnover of the enzyme and the species involved in the charge separation (electrons) is the same on each of two electron transfers to form QH_2 from Q at Q_c.

The position of cytochrome bc_1 redox centers in the membrane

Evidence derived from protein folding algorithms applied to primary sequence data for the cytochrome *b* polypeptide [62–64] has been interpreted as showing that the heme centers are approx. 2 nm from each other in the polypeptide. If the polypeptide is arranged such that both hemes are within the low dielectric of the membrane they would then span approx. 50–60% of the estimated 35–40 nm low dielectric hydrocarbon core [1,65]. Though this value is close to the fractional contribution of this reaction a few qualifications must be kept in mind when translating percentage values of any electrogenic contribution of a reaction into structural positions of the reactants. The $\Delta\psi$ generated by each electrogenic reaction is a function of the dielectric constant of the medium where the charge separation occurs and the distance of separation across this medium. The low dielectric medium is almost certainly not continuous in magnitude throughout the membrane. Moreover, the contribution of protein dielectric constant may introduce more complex discontinuities in the electrical capacitance of the membrane medium. Indeed, there is a puzzling variance between $\Delta\psi$ reporting phenomena and X-ray structural analysis in the determination of RC redox center positions in the membrane. Several studies relying on electrical potential measurements and assuming a homogeneous dielectric medium [4,13,66–68] imply a distance between cytochrome c_2 and the RC $[BChl]_2$ that is several-fold larger than would be expected from the X-ray structure analysis [65,69,70].

Our data may be interpreted to support a position for the heme span in the dielectric such that cytochrome b_L is near the periplasmic side of the membrane. It should be noted, however, that Kunz and Konstantinov [71] have proposed that cytochrome b_H is near the cytoplasmic surface and cytochrome b_L is buried in the low dielectric medium, based upon evidence with a water-soluble reductant, a possibility inconsistent with our data and with that of Ref. 20 (see Note added in proof).

EPR experiments using paramagnetic relaxation probes have shown that the 2Fe2S center is probably close to the periplasmic membrane surface [72]. A similar location has been assumed for cytochrome c_1 based upon its redox interaction with cytochrome c_2 and upon carotenoid bandshift data from a mutant of *Rhodobacter capsulatus* lacking cytochrome c_2 [73]. The data of this investigation support this positioning.

There are also data which indirectly address the location of the two quinone sites relative to the membrane surfaces. A number of inhibitor-re-

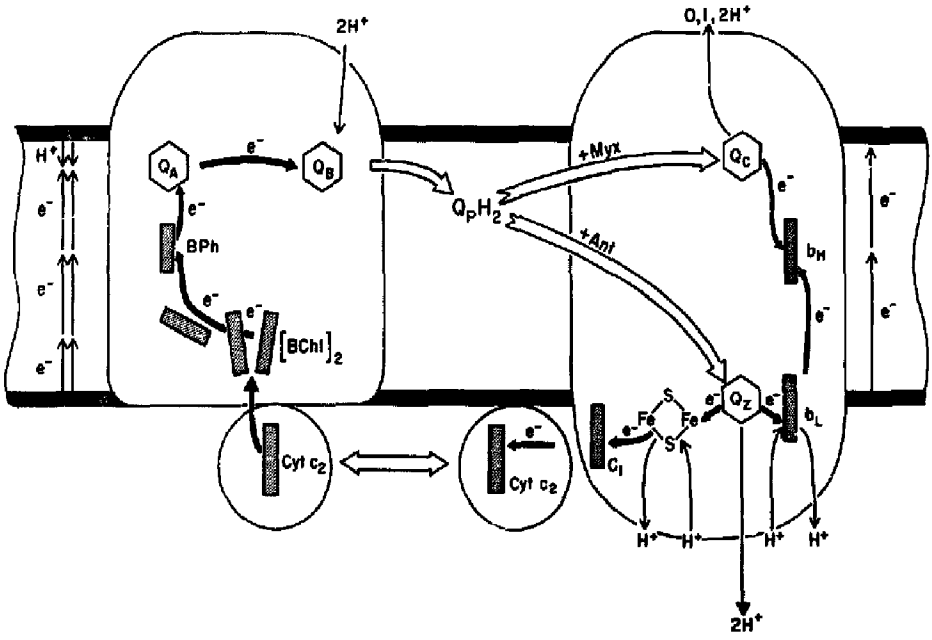

Fig. 9. Model showing the electrogenic reactions of the cyclic electron transfer system when each of the two routes to reduction of cytochrome b_H are isolated. The system is depicted with a single RC turning over and a partial reaction of cytochrome bc_1. The positioning of cytochrome b_H is based on a literal translation of percent electrogenic contribution into intramembrane distance. Cyt, cytochrome.

sistant mutants of cytochrome bc_1 from mouse [74,75], yeast [76] and *Rhodobacter capsulatus* (Daldal, F., personal communication) map in the cytochrome *b* polypeptide. A folding algorithm has been applied to the sequence of the cytochrome *b* polypeptide from a number of species [77] and the locations of amino acid substitutions conferring inhibitor resistance have been mapped relative to the membrane span. Those giving resistance to myxothiazol, stigmatellin and mucidin are grouped near the periplasmic face of the membrane (the cytoplasmic side of the mitochondrion) while those conferring antimycin, diuron or HQNO resistance are near the bacterial cytoplasmic side (or mitochondrial matrix side) of the membrane.

Taken together with these observations, our data are consistent with a simple model for the positions of the redox centers of cytochrome bc_1; (1) Q_z, cytochrome b_L, 2Fe2S and cytochrome c_1 are near the periplasmic edge of the dielectric; (2) cytochrome b_H is buried in the low dielectric medium between cytochrome b_L and the cytoplasmic surface; and (3) Q_c is on or near the cytoplasmic edge of the membrane low dielectric. These conclusions are summarized in Fig. 9 which shows the electrogenic reactions of the RC and the electrogenic contributions of each of the two reactions studied here which result in cytochrome b_H reduction in cytochrome bc_1.

As was the case for similar work done before the X-ray crystal structure of the RC, the results on cytochrome bc_1 outlined in this paper provide a valuable preliminary structural view. However, just as important, these studies reveal the relative contributions of each reaction to the overall electrogenic function of the complex as well as the chemical species moved through the membrane dielectric medium.

Consideration of the model of Fig. 9 when

operating in a physiological direction under uninhibited conditions suggests that the oxidation of the first QH_2 at the Q_z site will yield a much smaller $\Delta\psi$ than will oxidation of the second QH_2 which completes the cytochrome bc_1 turnover. This can be appreciated in a simplified description of the stepwise approach to steady state: after charge separation through the cytochrome c_2-RC span the $\Delta\psi$ is about 55 mV. The first QH_2 oxidized at the Q_z site will lead to cytochrome b_L reduction in a manner unaffected by the prevailing $\Delta\psi$, since there is no electrogenic component in this reaction sequence. Because of the relatively large ΔE_{m7} (+110 mV) between cytochrome b_L and cytochrome b_H, electron transfer from cytochrome b_L to cytochrome b_H across 60% of the membrane dielectric (i.e., apparent $\Delta E_{m7} = 110 - (55 \times 0.6) = 77$ mV) also should be essentially unaffected (less than 10%) by the opposing $\Delta\psi$ at this stage. In contrast, the ΔE_{m7} between cytochrome b_H and Q_c is near zero [29] and hence, electron transfer from cytochrome b_H to Q_c ($\Delta E_{m\,7} = 0$ mV) will not proceed very far against the $\Delta\psi$ and will add very little more to the cytochrome bc_1 charge separation.

The second QH_2 oxidized at the Q_z site will, as before, lead to cytochrome b_L reduction, adding nothing to $\Delta\psi$. With cytochrome b_H reduced after the oxidation of the first QH_2 the electrogenic cytochrome b_L to cytochrome b_H reaction will be blocked. However, in the equilibrium between cytochrome b_H and Q_c, every time Q_c^- is formed, cytochrome b_L has a chance to rereduce the oxidized cytochrome b_H. At this point the free energy between the reduced cytochrome b_H and Q_c^- is very large ($\Delta E_{m\,7}$ about 250 mV [29]) and will proceed to completion even against the prevailing $\Delta\psi$.

Two predictions emerge from this model.

(a) The ratio of $\Delta\psi$ generated following the first and second QH_2 oxidized will be about 60:140. In order to demonstrate this it will be necessary to resolve the oxidation of the first and second quinols at Q_z so that the state formed after the first oxidation is experimentally isolated and prevented from immediately being removed by subsequent (i.e., second) QH_2 oxidation. The difficulty here is that, as described above, the second oxidation is driven by a very much higher free energy than the first and is not kinetically separable. During the present study we have become aware of a surprisingly large statistical element governing the fate of QH_2 donated to the Q_{pool} by the RC. Several possibilities are evident, especially when QH_2 is limiting as is the case when Q_{pool} is oxidized before activation; these were the basis of the several corrections we made to the bandshifts in order to normalize the events with respect to electrogenic reactions. Extending this to careful accounting of the various states of the ensemble comprised of RC, cytochrome bc_1, Q and QH_2 and ferro/ferricytochrome c_2 may lead to a quantitative test of the model.

(b) After the first full turnover of cytochrome bc_1 (i.e., two QH_2 oxidized, one Q reduced, two charges moved across the membrane) the prevailing $\Delta\psi$ will be raised from 55 to 110 mV. As is well-known [4,5,13], the cyclic system can be turned over several times before $\Delta\psi$ becomes large enough to impede its electrogenic reaction. Of considerable importance to a complete mechanistic description of cytochrome bc_1 is a correlation of the effect of increasing $\Delta\psi$ on the kinetics of individual electrogenic reactions. This approach is currently under study.

Note added in proof (Received 16 August 1988)

Since submission of this manuscript, we have become aware of two additional studies which bear directly on the interpretation of data presented here. One has been in the literature for some time, the other has appeared since completion of our manuscript:

(1) Berden and Slater [78], studying the quenching of antimycin fluorescence after binding to cytochrome bc_1, calculated a distance of 17–21 Å between bound antimycin and cytochrome b_H. If we assume that antimycin occupies the same position as Q_c and that the 17 Å distance is perpendicular to the membrane plane, this result is consistent with the idea that electron transfer between these groups accounts for approx. 45% of the full charge separation catalyzed by the complex.

(2) Konstantinov and Popova [79] have proposed a model for cytochrome bc_1 where in Q_c and cytochrome b_H are arranged equilaterally near

the cytoplasmic membrane surface (mitochondrial matrix side) and Q_z and cytochrome b_L are buried in the membrane. This model invokes electrogenic proton movement from Q_z to the aqueous phase in an event coupled to cytochrome b_H oxidation. This model may, in part, be reconciled with the data of this paper if it is assumed that (a) the redox equilibria and associated protolytic reactions of cytochrome bc_1 are coupled across rather large intramembrane/intraprotein distances; (b) the protonation/deprotonations proposed to be electrogenic in the model are distinct from the redox-linked protons measured by conventional equilibrium redox potentiometry; (c) the electrogenic protons are associated with protein groups with pK values below 7.0, i.e., below the range studied with chromatophores in the present paper. Although there are data which may indirectly support the model proposing electrogenic protons we feel at present compelled to support the simplest interpretation of our data, i.e., that shown in Fig. 9.

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