The Electric Field Generated by Photosynthetic Reaction Center Induces Rapid Reversed Electron Transfer in the bc_1 Complex†

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ABSTRACT: The cytochrome bc_1 complex is the central enzyme of respiratory and photosynthetic electron-transfer chains. It couples the redox work of quinol oxidation and cytochrome reduction to the generation of a proton gradient needed for ATP synthesis. When the quinone processing Q_i- and Q_o-sites of the complex are inhibited by both antimycin and myxothiazol, the flash-induced kinetics of the *b*-heme chain, which transfers electrons between these sites, are also expected to be inhibited. However, we have observed in *Rhodobacter sphaeroides* chromatophores, that when a fraction of heme b_H is reduced, flash excitation induces fast (half-time ∼0.1 ms) oxidation of heme b_H, even in the presence of antimycin and myxothiazol. The sensitivity of this oxidation to ionophores and uncouplers, and the absence of any delay in the onset of this reaction, indicates that it is due to a reversal of electron transfer between b_L and b_H hemes, driven by the electrical field generated by the photosynthetic reaction center. In the presence of antimycin A, but absence of myxothiazol, the second and following flashes induce a similar (∼0.1 ms) transient oxidation of ∼10% of the cytochrome b_H reduced on the first flash. From the observed amplitude of the field-induced oxidation of heme b_H, we estimate that the equilibrium constant for sharing one electron between hemes b_L and b_H is 10−15 at pH 7. The small value of this equilibrium constant modifies our understanding of the thermodynamics of the Q-cycle, especially in the context of a dimeric structure of bc_1 complex.

The bc_1 complex (ubiquinol:cytochrome *c* oxidoreductase) plays a central role in free-energy transduction in biomembranes, and the proton-motive Q-cycle (*2*) is widely accepted as the underlying mechanism (reviewed in *1*). In this mechanism, one electron released upon quinol oxidation at the Q_o-site is transferred via the Rieske iron−sulfur protein (ISP)[1] to cytochrome *c*, while the second electron crosses the membrane via a chain of two hemes of the cyt *b* subunit, and reduces the occupant of the Q_i-site, either quinone or semiquinone, depending on the phase of the two-electron gate that operates at the site. Oxidation of two quinols at the Q_o-site is accompanied by release of four protons, while reduction of one quinone at site Q_i occurs with uptake of two protons, and transfer of two successive electrons across the membrane. In this way, the bc_1 complex generates an electrochemical proton gradient, which can be used for ATP synthesis, ion transport, and other kinds of work.

A critical component of the proton-motive Q-cycle mechanism is the *transmembrane* electron transfer between low- (b_L) and high-potential (b_H) hemes of cyt *b* (*2*). The structural arrangement of b_L and b_H hemes in an electron-transferring chain has been verified by X-ray structural analysis of bc_1 complex crystals from different sources (reviewed in *1*), which show heme b_L close to the Q_o-site and heme b_H close to the Q_i-site, as predicted. In mitochondria, West et al. (*3*) estimated that electron transfer between b_L and b_H occurred in less than 0.1 s. By measuring both low- and high-potential hemes in b_6f complex, Nitschke et al. (*4*) demonstrated a fast electron transfer between b_L and b_H. In both these systems, the kinetic resolution was limited by mixing. In chromatophores from the photosynthetic bacterium *Rb. sphaeroides*, fast reduction of b_H heme and oxidation of b_L heme on flash-activation of the photosynthetic chain had previously been directly demonstrated by Meinhardt and Crofts (*5*), who provided kinetic and spectral deconvolution of absorbance changes corresponding to both hemes. They observed a flash-induced reduction of cyt b_H with $t_{1/2}$ ∼1 ms when electron transfer from heme b_H was blocked by antimycin. In the absence of inhibitor, the small transient reduction of b_H allowed an estimate of the electron-transfer time, $t_{1/2}$ ∼100 μs, from heme b_H to the acceptor at the Q_i-site. A similar rate of reduction of heme b_L ($t_{1/2}$ ∼1 ms) was seen if heme b_H was previously reduced, but no significant transient when it was oxidized, indicating that electron transfer from b_L to b_H was much more rapid ($t_{1/2} \leq 100$ μs) than its reduction by the rate-determining step of the Q_o-site (*6*). However, activation of the bc_1 complex by the RC involves at least four intermediates ($c_2 \leftarrow c_1 \leftarrow$ ISP $\leftarrow QH_2 \rightarrow b_L \rightarrow b_H$), providing some uncertainty in the assignment of the time of electron transfer between b_L and b_H.

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; cyt, cytochrome; ISP, Rieske iron−sulfur protein; PMS, *N*-methylphenazonium methosulfate; RC, photosynthetic reaction center; Q_i, Q_o, quinone reducing and quinol oxidizing sites of bc_1 complex, respectively; b_L and b_H, low- and high-potential hemes of cytochrome *b*, respectively; $\Delta\psi$, transmembrane electric potential difference; *Rb.*, *Rhodobacter*.

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The regulation of energy transduction by the transmembrane proton gradient has obvious biological significance (*7*−*10*). Electron transfer in energy-transducing membranes is tightly coupled to generation of the proton gradient (*7*), and slowed by backpressure, leading to "control" of the rate, as in classical respiratory control (*8*). Discharge of the proton gradient, either physiologically (e.g., via ATP synthase) or artificially (by uncouplers), accelerates electron transport. This ensures that substrates are not oxidized until ATP is needed. The most obvious targets for backpressure from the proton gradient are stages in which charge is transferred across the membrane, which will "feel" the electrical component. In the bc_1 complex, such stages clearly involve transmembrane flux through low- and high-potential cytochrome *b* hemes, and effects of electric field on the reduction state of cytochromes have been studied for some time (*10*−*13*).

In this paper we show that the transmembrane electric field ($\Delta\psi$), generated by the reaction center in *Rb. sphaeroides* chromatophores, changes the poise of the electrogenic reactions of the bc_1 complex, allowing resolution of the kinetics of electron-transfer equilibration between the two *b*-hemes. Using this effect, we found that the equilibrium constant for transfer of one electron from b_L to b_H is much smaller than that calculated from midpoint redox potentials. This difference is attributed to the contribution of both delocalized fields and Coulombic interactions. These latter depend on occupancy of the heme centers, and may lead to cooperative interaction between the two monomers.

MATERIALS AND METHODS

Growth of Cells and Isolation of Chromatophores. Cells of the Ga(pC2P404.1) strain (cyt c_2 superproducer, *14*) of *Rb. sphaeroides* Ga were grown photosynthetically at 30 °C in Sistrom's medium in the presence of 1 μg/mL tetracycline using far-red filters to minimize antibiotic photooxidation. Chromatophores were isolated by differential centrifugation as described elsewhere (*15*). The superproducer strain was used to minimize delays in transfer of an oxidizing equivalent from RC to the bc_1 complex.

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 provided by xenon flash (~5 μs half-duration). The ambient redox potential in the cuvette was adjusted by adding small amounts of $K_3Fe(CN)_6$ or $Na_2S_2O_4$, and the suspension was kept anaerobic by a constant flow of argon gas above the sample. The following buffer A was used in all experiments: 50 mM MOPS (pH 7.0), 100 mM KCl, 1 mM Fe-EDTA (redox buffer), and 1 mM NaCN. The redox changes of cyt c_1 plus cyt c_2 ($=c_{tot}$) were measured at 551−542 nm ($\epsilon^{mM} = 20\ mM^{-1}\ cm^{-1}$; *16*, *17*). Cytochrome b_H reduction was measured at 561−569 nm ($\epsilon^{mM} = 20\ mM^{-1}\ cm^{-1}$; *18*) in the presence of antimycin. The RC concentration was estimated at 542 nm ($\epsilon^{mM} = 10\ mM^{-1}\ cm^{-1}$; *16*, *19*). The kinetics of cyt b_L redox changes were obtained by taking the difference in the kinetics measured at 566 and 575 nm, minus 0.5 change measured at 561 minus 569 nm (*5*).

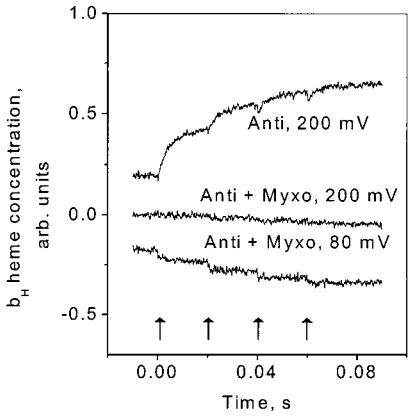

FIGURE 1: Kinetic traces of cytochrome b_H on flash excitation (arrows) of *Rb. sphaeroides* chromatophores. Chromatophores were suspended in buffer A with 10 μM PMS, and 2 μM methylene blue. Traces shown are the average of 4, with 10 s (top and center traces) and 50 s (bottom trace) between measurements. The redox potential was 200 mV (top and center) and 80 mV (bottom). Where indicated, the concentrations of antimycin A and myxothiazol were 7 and 2 μM, respectively.

To minimize interference from overlapping absorbance changes, we also determined the kinetics of hemes b_H and b_L from kinetic traces measured at 2−4 nm steps over the wavelength range from 540 to 575 nm, using published spectra for c_2, c_1, P870, b_L, and b_H (*20*). The least-squares method used was similar to that described in (*21*). Curves shown in Figures 1−3 were obtained by using such a procedure. Both methods gave similar results. In some cases, absorbance changes of cytochromes were corrected for electrochromic changes using the method suggested by Venturoli et al. (*23*). In general, such correction did not significantly modify the results of deconvolution. The electrogenic activity of the photosynthetic chain was monitored through the carotenoid electrochromic bandshift at 503 nm.

Reagents. Antimycin A, myxothiazol, PMS, CCCP, and MOPS were obtained from Sigma Chemical Co. Inhibitors were dissolved in ethanol and stored at −20 °C.

RESULTS

Flash-Induced Kinetics of b_H at 200 and 80 mV. At ambient redox potential (E_h) of 200 mV, all high-potential components of the bc_1 complex (c_1, ISP) are reduced, while b_L, b_H, and the ubiquinone pool are oxidized. Flash activation of the RC under these conditions leads to the generation of oxidized cyt c_2 and QH_2, which are the substrates for the bc_1 complex. Figure 1 shows the flash-induced kinetics of heme b_H in *Rb. sphaeroides* chromatophores in the presence of antimycin A, an inhibitor of the Q_i-site. In agreement with previous studies (*18*), the reduction of b_H heme occurs with a half-time of ~10 ms. Further addition of myxothiazol (Figure 1, center) leads to complete inhibition of the flash-induced reduction of cyt b_H. Thus, when the Q_i- and Q_o-sites of the complex are inhibited by both antimycin and myxothiazol, electron transfer linked to the quinone processing sites is completely blocked, as previously found (cf. *15*, *17*, *22*). We found, however, that when these inhibited chromatophores were poised at potentials where a significant fraction of heme b_H ($E_{m,7} \sim 50$ mV) is reduced in the dark,

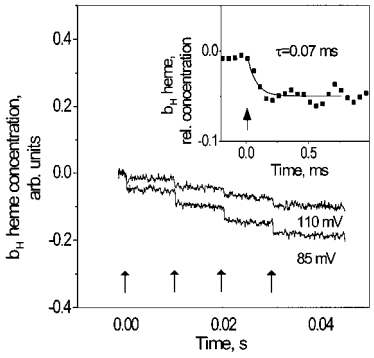

FIGURE 2: Flash-induced kinetics of b_H in the presence of 7 μM antimycin and 2 μM myxothiazol at different redox potentials. Chromatophores were suspended in buffer A with 4 μM PMS, and 0.3 mM sodium ascorbate. Traces shown are the average of 4, with 40 s between measurements. The instrument response time was 33 μs. The redox potential was 85 and 110 mV as indicated. The inset shows the exponential fit of b_H heme oxidation induced by the first flash at E_h = 85 mV.

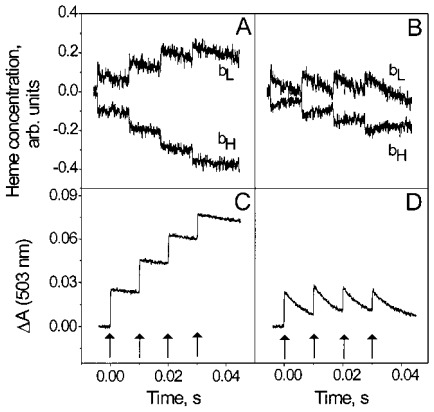

FIGURE 3: Flash-induced oxidation of b_H and b_L (A, B) and carotenoid change (C, D) of *Rb. sphaeroides* chromatophores, in the absence (A, C) and presence (B, D) of 7 μM CCCP. Chromatophores were suspended in buffer A with 4 μM PMS, and 0.3 mM sodium ascorbate. Antimycin A (7 μM) and myxothiazol (2 μM) were present to inhibit Q_i- and Q_o-sites. Traces shown are the average of 4, with 50 s between measurements. E_h was 85 mV.

actinic flashes induced a rapid *oxidation* ($t_{1/2}$ ~0.1 ms) of heme b_H (Figure 1, bottom). The amplitude of the flash-induced oxidation increased progressively as the E_h was lowered (Figure 2). Moreover, in parallel with flash-induced oxidation of the b_H heme, we observed a fast *reduction* of the b_L heme (Figure 3A).

Because these changes were observed in the presence of antimycin A and myxothiazol, when both Q_o- and Q_i-sites are inactive, and because they were much faster (~0.1 ms) than the time of electron exchange between the RC and the bc_1 complex, they most likely reflect the influence of $\Delta\psi$, which has previously been shown to change the electron equilibrium between b_L and b_H hemes (*12*, *13*).

Effect of Uncouplers. To check this hypothesis, we measured parallel changes in the electrochromic bandshift of carotenoids (carotenoid change), and the redox state of cyt b_H at an E_h where b_H was partially reduced before the flash. As required by the hypothesis, the oxidation of b_H heme and the reduction of b_L heme are both sensitive to the $\Delta\psi$ (compare panels A and B of Figure 3). The kinetics of re-reduction of b_H heme also closely followed the discharge of the $\Delta\psi$ (compare panels B and D of Figure 3). The observed

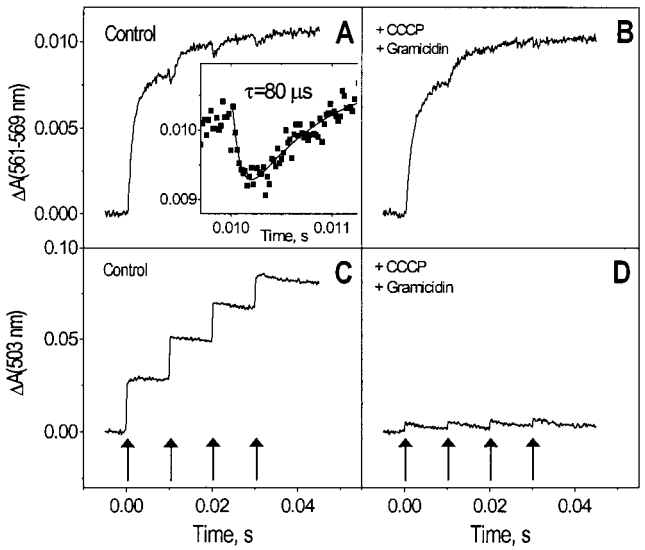

FIGURE 4: Kinetic traces of cytochrome b_H (A, B), and carotenoid change (C, D) of *Rb. sphaeroides* chromatophores, in the presence of 7 μM antimycin A. Chromatophores were suspended in buffer A with 4 μM PMS, and 0.3 mM sodium ascorbate. Traces shown are the average of 16, with 20 s between measurements. E_h was 140 mV (A, C) and 170 mV (B, D). Where indicated, the concentrations for CCCP and gramicidin were 3.3 and 40 μM, correspondingly. The inset in panel A shows kinetics of the second flash-induced oxidation of the b_H heme obtained as the average of 90 traces recorded with an instrument response time of 8 μs.

trend in both b_L and b_H is possibly due to their slow oxidation, via b_L, by redox mediator.

The sensitivity of cyt *b* oxidation to ionophores and uncouplers (Figure 3), the parallel oxidation of b_H heme and reduction of b_L heme (Figure 3), the absence of any delay in the onset of this reaction (Figure 2, inset), and the increase of the b_H oxidation on lowering the E_h (Figure 2) all strongly indicate that this oxidation is due to a reversal of electron transfer between b_L and b_H hemes, driven by the electrical field generated by the RC. The lifetime of this field-induced oxidation of heme b_H is ~0.1 ms (Figure 2).

Transient Oxidation of b_H Heme Induced by a Second Flash in the Presence of Antimycin Alone. The flash-induced oxidation of b_H at E_h < 110 mV with both antimycin and myxothiazol present was seen only because b_H was partially reduced before the flash. Reduction of b_H heme before the testing flash can be achieved in the presence of antimycin A alone (in the *absence* of myxothiazol). Indeed, in the presence of antimycin, the first flash induces almost complete reduction of heme b_H (Figure 4A). The subsequent flashes induce a fast (~0.1 ms) oxidation (dip) of about 10% of the cyt b_H reduced after the first flash (Figure 1, top, and Figure 4A). It is important to note that this fast phase of oxidation (dip) is seen only after the second and following flashes and was absent after the first flash, in good correspondence with the suggestion that this phase is due to the field-induced transfer of electrons from b_H to b_L. Addition of uncouplers decreased the amplitude of the dip due to b_H oxidation after the second and following flashes (compare panels A and B in Figure 4), in agreement with its dependence on the flash-induced electrical field. The dip due to heme b_H oxidation occurs before reducing and oxidizing equivalents from the RC arrive at the bc_1 complex and initiate the chemistry at the Q_o-site, and subsequent heme b_H reduction.

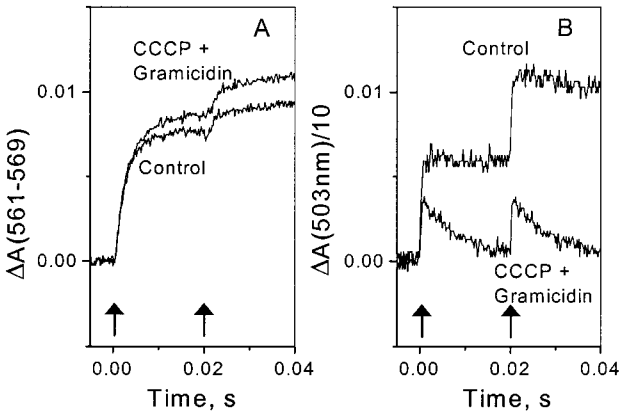

FIGURE 5: Effect of uncouplers on the kinetics of b_H heme reduction (A) and electrochromic shift of carotenoids (B) in the presence of 7 μM antimycin A. *Rb. sphaeroides* chromatophores were suspended in buffer A with 2 μM PMS and 0.5 mM sodium ascorbate. Traces shown are the average of 4, with 30 s between measurements. The redox potential was 140 mV. Where indicated, concentrations for CCCP and gramicidin were 3.3 and 20 μM, respectively.

Dependence of the Amplitude of Heme b_H Reduction on the Proton Gradient. The observations above show that the fast (~0.1 ms) kinetic change in the presence of both bc_1 complex inhibitors corresponds to field-induced electron transfer from b_H to b_L. Similarly, we concluded that the fast transient b_H oxidation seen after the second flash, when antimycin alone is present, also has a similar origin. A further indication of the change in equilibrium constant induced by the field is the observation that the amplitude of the flash-induced cyt *b* reduction increased after addition of uncouplers (Figure 5), indicating that the amplitude seen under coupled conditions was lowered by backpressure from the $\Delta\psi$.

DISCUSSION

Nature of the Flash-Induced b_H Changes in the Presence of both Antimycin A and Myxothiazol. While it is customary to assign the changes measured at 561−569 nm to b_H (*16*), these wavelengths are overlapped by electrochromic responses of carotenoids and bacteriochlorophylls (*23*), and the absorbance changes of other redox components. Plotting the data using difference kinetics at fixed wavelength, to demonstrate the flash-induced oxidation of b_H and reduction of b_L in the presence of antimycin and myxothiazol, revealed spectral overlap of the small field-induced *b*-cytochrome redox changes by the relatively large changes of cytochrome c_{tot} and the electrochromic carotenoid change which obscured the kinetics. We therefore develop a method of spectral deconvolution using a least-squares method, which corrected for these overlapping components, and also applied the correction for the electrochromic changes suggested by Venturoli et al. (*23*). Even with these precautions, some residual contribution of electrochromic responses may contaminate changes attributed to heme b_L, and we have therefore based estimations of the equilibrium constant on the changes of heme b_H, which are least affected by such interference.

The following observations support the interpretation of flash-induced changes of b_H in the presence of both antimycin and myxothiazol as the oxidation of b_H (and reduction of b_L heme) induced by the electric field generated by RC: (i) The flash-induced changes of b_H are seen only when b_H is reduced before the flash, and the amplitude of the change increases with increase in the fraction of b_H reduced before the flash (Figure 2). (ii) The flash-induced changes of b_H are sensitive to uncouplers (Figure 3). (iii) This phase is faster than electron exchange between RC and cyt *b* in the bc_1 complex (Figure 2). Thus, we conclude that the observed kinetics of b_H heme oxidation with τ ~0.1 ms correspond to electron transfer from b_H to b_L induced by $\Delta\psi$ generated by RC turnover (~5 μs, as determined by the xenon flash duration).

Nature of the Dip in the Kinetics of Cytochrome b Reduction Induced after the Second and Following Flashes. The transient dip seen after the second flash is sensitive to uncouplers, indicating a significant role of the electric field in the apparent value of the equilibrium constant of electron transfer from b_L to b_H. It is important to note that the "dip" in kinetics of cyt *b* reduction is seen only after the second and subsequent flashes (Figure 4). This observation provides an internal control, since it shows that the transient is dependent on the presence of reduced b_H (created after the first flash). Only then was the electric field able to induce oxidation, as seen after the second and subsequent flashes. We also excluded the probability that the observed changes are due to the electrochromic shifts of the absorbance spectra of cytochrome, by inclusion in the deconvolution the derivative of the b_H spectrum. This only slightly modified the kinetic changes of reduced b_H (<10%, and only in the presence of antimycin A alone).

Equilibrium Constant for One-Electron Transfer from b_L to b_H in the Presence of both Antimycin and Myxothiazol. The equilibrium constant for one-electron transfer between b_L and b_H hemes depends on the $\Delta\psi$ and can be estimated from measurements of the flash-induced oxidation of cyt b_H in the presence of both antimycin and myxothiazol. At redox potentials where b_L is completely oxidized and b_H is partially reduced ($50 \leq E_h \leq 120$ mV), the electric field induces redistribution of electrons between b_L and b_H, as follows: $b_L b_H^- \Leftrightarrow b_L^- b_H$. In this case, the oxidation of b_H is equivalent to the reduction of b_L and vice versa. The equilibrium constant can be estimated from the equation:

$$K = (1 - [b_H])/[b_H] \quad (1)$$

where $[b_H]$ is the fraction of b_H heme oxidized after the flash, and we assume that all electrons that leave b_H arrive at b_L. This fraction can be estimated by normalizing the absorbance changes corresponding to oxidation of b_H heme to the total concentration of b_H.

We can assume exponential dependence of the equilibrium constant $K = k_1/k_{-1}$ on $\Delta\psi$ (*25*). Thus, because the amplitude of the carotenoid change at 503 nm is proportional to $\Delta\psi$ (*27*), the equilibrium constant K should be exponentially proportional to this change. This allows us to estimate the equilibrium constant at $\Delta\psi = 0$. Figure 6B shows the dependence of K calculated from eq 1 as a function of the carotenoid change, measured in parallel to b_H oxidation. The equilibrium constant is about 3 after the first flash and below 1 after the third and following flashes. Using a linear fit of the dependence log(K) on ΔA(503 nm), we can estimate that the equilibrium constant is about 10 at $\Delta\psi = 0$.

Estimation of the Apparent Equilibrium Constant of Electron Transfer from b_L to b_H in the Presence of Antimycin

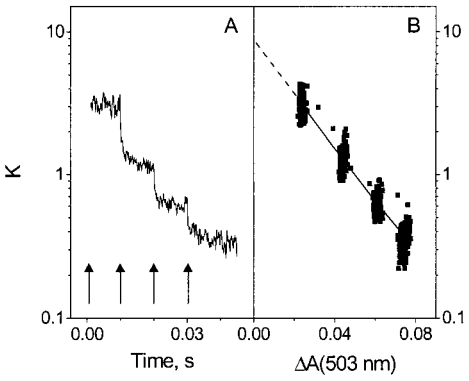

FIGURE 6: Dependence of the equilibrium constant of one-electron partitioning between b_L and b_H on the time (A) and on the electrochromic shift of carotenoids (B), calculated from eq 1 using data shown in Figure 3A,C. Only points taken after 1 ms after the first flash are shown.

Only. In addition to the approach used above, there are at least two independent, but less direct methods for estimating the value of the *apparent* equilibrium constant of electron transfer from b_L to b_H in the presence of antimycin only: (i) using the stimulation of the amplitude of b_H reduction on the first flash by uncouplers, and (ii) using the amplitude of the 'dip' induced by the second flash. For method (i), Figure 5 shows that the ratio of amplitudes of heme b_H reduction with and without uncouplers is about 1.12, yielding an apparent equilibrium constant $K^{app} \sim 13$. Using the second approach (ii), Figure 5B shows that the second flash induces an electrochromic shift that is 0.74 of that induced by the first flash, corresponding to about 37 mV. The ratio of cyt *b* reduction after the first flash (measured just before the second flash) and *immediately* after the second flash is about 1.1. From here we find that the apparent equilibrium constant $K^{app} \sim 22$ (values in the range 16−25 were found with different preparations). Thus, both methods give similar estimates for the apparent equilibrium constant of electron transfer from b_L to b_H at $\Delta\psi = 0$, yielding a range between 10 and 25 (at pH 7).

Although the apparent equilibrium constant of 13−25 in the presence of antimycin alone is close to the value of $K \approx 10$ found in the presence of both antimycin and myxothiazol, we must consider possible artifacts. Myxothiazol binding changes the spectrum, and has a small effect on the E_m, of heme b_L (*17*, *20*, *28*), so direct interaction with the inhibitor, including a change in E_m, could account for the small difference. It is also possible that the value derived from measurement of the amplitude of the "dip" could be influenced by interaction with the Q_o-site, which, in the absence of myxothiazol, is chemically connected to the *b*-heme chain. Oxidation of QH_2 by the ferricytochrome c_2 generated in the second flash is not likely to contribute, since the measurements of the "dip" were made during the lag before redox equivalents from the RC reach the Q_o-site. However, the degree of reduction of heme b_H had reached a steady value prior to the second flash, so the reaction at the Q_o-site was at quasi-equilibrium. Pressure from the poise of the Q_o-site reaction might be expected to increase the work required to transfer an electron in reverse, and could contribute to the difference between the values seen with and without myxothiazol.

Estimation of the Rate Constant of Electron Transfer from b_L to b_H. In the presence of antimycin A and myxothiazol, the electric field effect can be seen only in the fraction of bc_1 complexes that has only one electron per monomer. The kinetics of electron transfer in such "isolated" systems depend on the sum of forward and backward rate constants (see, e.g., *24*). When the equilibrium constant, $K = k_1/k_{-1}$, is significantly greater than 1, $k^{obs} = k_1 + k_{-1}$ is dominated by k_1. For $K = 10$, $\tau \sim 80$ μs, $k_1 \sim 1.1 \times 10^4$ s^{-1}, and $k_{-1} \sim 1.1 \times 10^3$ s^{-1}.

The electrical potential ($\Delta\psi$) applied across the membrane will decrease the forward rate constant of electron transfer from b_L to b_H (k_1) and increase the backward rate constant of electron transfer from b_H to b_L (k_{-1}). As a first approximation, we can assume exponential dependence of the rate constants on $\Delta\psi$ (*25*):

$$k_1 = k_1^0 \cdot e^{-(\alpha\Delta\psi F/2RT)},\ k_{-1} = k_{-1}^0 \cdot e^{(\alpha\Delta\psi F/2RT)} \qquad (2)$$

Here R is the gas constant, T is the absolute temperature, α is the fraction of $\Delta\psi$ applied between b_H and b_L, and k_1^0 and k_{-1}^0 are the respective rate constants at $\Delta\psi = 0$. Because the center to center distance between b_L and b_H is about 20 Å, we have assumed a value of $\alpha \approx 0.5$, but this could be refined on further analysis.

The $\Delta\psi$ generated by a single saturating flash is about 50 mV (*26*), of which only approximately half is applied between the *b* hemes. In this case, we can estimate that $k_1 \approx k_1^0 \times 0.6$; i.e., the new rate constant is about half of that at $\Delta\psi = 0$ ($k_1^0 \sim 1.1 \times 10^4$ s^{-1}). The measured kinetics of b_H oxidation show a lifetime of ~80 μs (see inset in Figure 4A). This value is within the range previously estimated from the small transient kinetics of heme b_L reduction under uncoupled conditions, when heme b_H is oxidized before the flash (*5*).

Different Contributions to $\Delta E_{m,7}$. The equilibrium constant of electron transfer from b_L to b_H found here ($K \sim 10-25$) is in sharp contrast to estimations of $K \sim 200$, based on E_m values from equilibrium redox titrations (−90 and 40−50 mV for hemes b_L and b_H, respectively; *17*, *29*). Since the values for K in the presence and absence of myxothiazol did not differ greatly, the difference in K cannot be ascribed solely to a modification of the E_m of heme b_L by myxothiazol (*28*). In discussing the difference, it is helpful to recognize several conditions of the *b*-heme chain and its electron occupancy. We can represent the work for reduction of heme b_L under the different conditions as follows, with the different terms discussed below:

$$\Delta G^{app} = \Delta G^\circ + \Delta G^{field} + \Delta G_m^{Coulombic} + \Delta G_d^{Coulombic}$$

For convenience, we express values in electrical units.

(a) Under uncoupled conditions (no electrical gradient across the membrane), the first electron in the *b*-heme chain will distribute between the two centers according to their intrinsic electron affinities, ΔG°.

(b) Introduction of a second electron into the chain will require additional work because of the Coulombic repulsion due to the charge of the first electron. This is the condition under which the redox potential of heme b_L is normally measured. The ΔG calculated from the E_m difference will be the sum of ΔG values due to the intrinsic affinity

difference, and to the electrostatic repulsion: $\Delta G^{app} = \Delta G^{\circ} + \Delta G_m^{Coulombic}$. This is also the condition under which reduction of heme b_L, following flash excitation under uncoupled conditions, was measured by Meinhardt and Crofts (*5*), and interpreted in the context of the E_m value determined by redox titration.

(c) Under coupled conditions, the distribution of the first electron between centers will be biased by the vectorial effect of the electric field provided by the membrane potential. Effectively, the change in relative redox potential will give rise to redistribution of the electron between b_L and b_H: $\Delta G^{app} = \Delta G^{\circ} + \Delta G^{field}$. This is the situation under which the value for K is measured in the experiments reported here. This can be seen most clearly when the *b*-heme chain is "isolated" by addition of both inhibitors, and the perturbation by the field reveals the equilibrium constant due to the intrinsic differences in electron affinity (Figures 1−3). The effect is also obvious from the increased amplitude of heme b_H reduction after the first flash in the presence of uncouplers (Figure 5).

(d) The discussion above takes no account of the dimeric nature of the bc_1 complex. In the dimer, the two heme b_L centers are almost as close (21.3 Å between heme irons) as the b_L and b_H hemes of the monomer (20.5 Å separation), and electron distribution between both pairs of hemes will likely be constrained by similar Coulombic effects ($\Delta G_d^{Coulombic}$). A weaker Coulombic interaction between monomers will also likely be felt between the b_H hemes (33.5 Å separation) (*1*).

The experiments reported here allow us to estimate values for the different contributions to the E_m values for b_L and b_H hemes observed in equilibrium titration. The intrinsic electron affinity difference, ΔG°, is given by the equilibrium constant determined here ($K \sim 10-25$), and contributes about 60−70 mV of the ∼140 mV (pH 7) difference between the E_m values. This allows estimation of what the E_m value for heme b_L would be in the absence of the Coulombic effect, giving an E_m of about −10 to −35 mV. This is close to the value $E_m \sim -15$ mV reported for heme b_L in a strain in which incorporation of heme b_H was prevented by mutation of a histidine ligand (*29*). Because of the possibility of additional effects arising from mutation, the similarity between these values should be treated with caution. However, the simplistic interpretation would suggest that the Coulombic effects on the E_m of the b_L heme could be as high as −80 mV, and this would represent the sum of monomeric ($\Delta G_m^{Coulombic}$) and dimeric ($\Delta G_d^{Coulombic}$) contributions, with the former term likely to be the most important (but see below for interactions when both b_L are reduced).

We note that the contribution of similar terms would be expected to modulate the electron-transfer reaction between the Q_i-site occupant and heme b_H. That this is the case can be seen in preliminary reports of an increased reduction of heme b_H observed under coupled conditions when the Q_i-site operates in reverse (*32*, *33*).

Mechanism of bc_1 Complex Turnover under Coupled Conditions. The small value for the equilibrium constant for one-electron sharing between b_L and b_H hemes could lead to a significant decrease in the rate of the electron transfer in the presence of a fully developed $\Delta\psi$. Indeed, the apparent equilibrium constant might be expected to decrease from about ≈10 ($\Delta\psi = 0$ mV) to ≈0.1 ($\Delta\psi = 240$ mV), so that a single electron in the chain would be predominantly located on the b_L heme. Whether this would inhibit QH_2 oxidation at the Q_o-site will depend on parameters for which limited information is available. The rate of reduction of heme b_L by Q_o semiquinone depends on the concentration of Q_o semiquinone and oxidized b_L (*31*). With heme b_L fully oxidized, this rate does not appear to be limiting, even when heme b_H is reduced before flash activation (*6*, *31*).

Reduction of b_L by reversal of electron transfer is well-established (cf. *34*), so additional backpressure from the proton gradient can certainly reverse the electrogenic reactions of the bc_1 complex. However, under coupled forward electron transfer, the constraint from $\Delta\psi$ is balanced by the driving force provided by the redox drop between QH_2 and cyt *c*, which increases as the "crossover" effect develops (*8*). Chen and Crofts (*35*) measured the poise of the redox centers in the coupled steady state, and showed that all the cytochromes, and the quinone pool, were close to the half-reduced state, suggesting that the chain operates at maximal kinetic efficiency. When the quinone pool is partly oxidized, the overall reaction is relatively rapid due to oxidation of b_H heme at site Q_i, leading to rapid b_L oxidation. However, if the quinone pool is fully reduced, this pulling mechanism is not available until a quinone generated at the Q_o-site can diffuse to the Q_i-site, and the rate of QH_2 oxidation can slow significantly (*5*).

We now consider the possibility that under these conditions, due to the electrostatic interactions between the *two low-potential hemes* in a dimer, the redox potential for the transition $[b_L b_L^-] \rightarrow [b_L^- b_L^-]$ is lower than that for the first electron $[b_L b_L] \rightarrow [b_L b_L^-]$. Assuming an effective dielectric constant ∼10, the electrostatic interaction between hemes 21 Å apart could approach ∼70 mV. A large value for this Coulombic interaction would result in a separation of the titration curve for heme b_L into two components centered at the mean of their values, but simulations show that the departure from a single component titration due to separation by as much as 50 mV would be within the error of most data reported (*20*, *28*, *29*). However, even a relatively weak interaction would have the effect of keeping the b_L of one monomer more oxidized than the other, resulting in a faster rate of electron transport, and this might be of importance in minimizing superoxide production as a result of Q_o semiquinone oxidation by oxygen under coupled conditions.

CONCLUSIONS

In this paper we have taken advantage of the rapid production of an electric field by charge separation in the *reaction center* to measure the influence of membrane potential on the reactions of the bc_1 complex, and have provided the first quantitative analysis of these effects. From the modulation of the electron transfer in the bc_1 complex by electric potential, we were able for the first time to estimate both the rate and equilibrium constants for electron transfer between the low- and high-potential hemes in situ. The time of electron transfer between b_L and b_H was found to be ∼0.1 ms. It was also found that the equilibrium constant for transfer of one electron from b_L to b_H is 1 order of magnitude lower than previous estimation based on values of midpoint potentials for b_L and b_H, leading to the necessity

of reevaluation of the thermodynamics of the Q-cycle. The method of fast application of electric potential, applied here to the bc_1 complex, can in principle be used more widely for the analysis of electrogenic reactions in other membrane proteins.

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