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CYTOCHROME *b* OXIDATION AND REDUCTION REACTIONS IN THE UBIQUINONE-CYTOCHROME b/c_2 OXIDOREDUCTASE FROM *RHODOPSEUDOMONAS SPHAEROIDES*

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Summary

1. The kinetics of cytochrome *b* reduction and oxidation in the ubiquinone-cytochrome b/c_2 oxidoreductase of chromatophores from *Rhodopseudomonas sphaeroides* Ga have been measured both in the presence and absence of antimycin, after subtraction of contributions due to absorption changes from cytochrome c_2, the oxidized bacteriochlorophyll dimer of the reaction center, and a red shift of the antenna bacteriochlorophyll.

2. A small red shift of the antenna bacteriochlorophyll band centered at 589 nm has been identified and found to be kinetically similar to the carotenoid bandshift.

3. Antimycin inhibits the oxidation of ferrocytochrome *b* under all conditions; it also stimulates the amount of single flash activated cytochrome *b* reduction 3- to 4-fold under certain if not all conditions.

4. A maximum of approximately 0.6 cytochrome *b*-560 ($E_{m(7)} = 50$ mV, $n = 1$, previously cytochrome b_{50}) hemes per reaction center are reduced following activating flashes. This ratio suggests that there is one cytochrome *b*-560 heme functional per ubiquinone-cytochrome b/c_2 oxidoreductase.

5. Under the experimental conditions used here, only cytochrome *b*-560 is observed functional in cyclic electron transfer.

6. We describe the existence of three distinct states of reduction of the ubiquinone-cytochrome b/c_2 oxidoreductase which can be established before acti-

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Abbreviation: BChl, bacteriochlorophyll.

vation, and result in markedly different reaction sequences involving cytochrome *b* after the flash activation. Poising such that the special ubiquinone (Q_z) is reduced and cytochrome *b*-560 is oxidized yields the conditions for optimal flash activated electron transfer rates through the ubiquinone-cytochrome b/c_2 oxidoreductase. However when the ambient redox state is lowered to reduce cytochrome *b*-560 or raised to oxidize Q_z, single turnover flash induced electron transfer through the ubiquinone-cytochrome b/c_2 oxidoreductase appears impeded; the points of the impediment are tentatively identified with the electron transfer step from the reduced secondary quinone (Q_{II}) of the reaction center to ferricytochrome *b*-560 and from the ferrocytochrome *b*-560 to oxidized Q_z, respectively.

Introduction

The functional unit of photosynthetic electron transfer and proton translocation in chromatophores from *Rhodopseudomonas sphaeroides* consists of the reaction center protein, and the ubiquinone-cytochrome b/c_2 oxidoreductase that together form a cyclic system [1]. While the initial light activated charge separation and generation of a strong oxidant and a strong reductant takes place in the reaction center protein, it is the job of the ubiquinone-cytochrome b/c_2 oxidoreductase to complete the electron transfer cycle. Of three *b* cytochromes first identified with the ubiquinone-cytochrome b/c_2 oxidoreductase [2] only cytochrome *b*-560 ($Em_7 = 50$ mV, $n = 1$, previously cytochrome b_{50}) has received convincing support for an immediate role in cyclic photosynthetic electron and proton transfer reactions.

Earlier work has suggested that the secondary quinone (Q_{II}) of the reaction center is the reductant of cytochrome *b*-560, and that cytochrome *b*-560 functions as an electron transfer agent between Q_{II} and cytochrome c_2 [3], although recent results indicate that this cytochrome plays a larger role in a more flexible electron transfer pathway: It is now recognized that cytochrome *b* reduction can also occur via an in situ 'oxidant induced reduction'; in this pathway, it has been proposed that flash oxidized cytochrome c_2 is reduced (via the Rieske iron sulfur cluster [4,5]) by a special reduced ubiquinone species (designated Q_zH_2) to yield an unstable intermediate, $Q_z \cdot H$, which in turn reduces ferricytochrome *b* to produce Q_z [6]. In addition to its role in electron transfer, the cytochrome *b*-560 is also functional as a proton translocating agent [7—9].

Most of the previous studies on cytochrome *b* in *Rps. sphaeroides* chromatophores have been carried out in the presence of antimycin which prevents prompt ferrocytochrome *b* oxidation and therefore simplifies experimentation [3,6,9,10]. However, in order to study the oxidation kinetics of ferrocytochrome *b*, and investigate the role of the cytochrome in the energy coupled reactions of the uninhibited ubiquinone-cytochrome b/c_2 oxidoreductase, it has become important to examine more closely its reactions in the absence of antimycin. Since the absorption changes associated with cytochrome *b* reactions in the absence of antimycin are not as large as in its presence, we must first assess possible absorbance contributions to spectroscopic analysis made in the cyto-

chrome *b* α-band region by other chromatophore components.

The need to recognize the importance of the several minor but, under certain conditions, collectively significant spectral interferences to cytochrome *b* measurements was stressed in similar work in *Rhodopseudomonas capsulata* and *Rps. sphaeroides* [11]. In this paper, by subtracting from wavelengths used to measure cytochrome *b*, the contributions accompanying the oxidation-reduction of cytochrome c_2 and the bacteriochlorophyll dimer $(BChl)_2$, and a spectral red shift on the antenna bacteriochlorophyll we detail the flash activated reduction and oxidation kinetics of this cytochrome both in the presence and absence of antimycin.

Materials and Methods

Experimental conditions

Preparation of chromatophores [12] and techniques of flash kinetic spectrophotometry and redox potentiometry have been previously described [13]. Most of the experiments in this paper were carried out at pH 6.0 and redox potential (E_h) values of 50, 150, 250, and 380 mV. These potentials were chosen in order to study the effects of initial redox poise of the ubiquinone-cytochrome b/c_2 components by selecting E_h values which differentiate between several key components of the cycle. The pH chosen is well below the p*K* values on cytochrome *b*-560 and the proton binding reactions [7—9]. The initial redox states of the components of the reaction center and the ubiquinone-cytochrome b/c_2 oxidoreductase at pH 6.0 are outlined below:

a. At a redox potential of 50 mV, the reaction center primary quinone (Q_I) is 50% reduced, cytochrome *b*-560 is 90% reduced, and the Q pool, Q_z, another cytochrome *b* ($E_{m(7)}$ = 155 mV), cytochrome c_2, the Rieske iron sulfur cluster, and the reaction center $(BChl)_2$ are more than 99% reduced.

b. At a redox potential of 150 mV, Q_I is more than 95% oxidized, cytochrome *b*-560 is 83% oxidized, the Q pool is 50% reduced, cytochrome *b* (155 mV) and Q_z, cytochrome c_2, the Rieske iron sulfur cluster, and the reaction center $(BChl)_2$ are more than 99% reduced.

c. At a redox potential of 250 mV, Q_I, Q_z, the Q pool and cytochrome *b*-560 are more than 95% oxidized, cytochrome *b* (155 mV) is 78% oxidized, cytochrome c_2 and the Rieske iron sulfur cluster are more than 80% reduced, and the reaction center $(BChl)_2$ is more than 99% reduced.

d. At a redox potential of 380 mV, Q_I, Q_z, the Q pool, cytochromes *b*-560 and cytochrome *b* (155 mV) are all >99% oxidized, cytochrome c_2 and the Rieske iron sulfur cluster are more than 95% oxidized, and the reaction center $(BChl)_2$ is 95% reduced.

The redox states of the reaction center Q_{II} at the above redox potentials are not included here, but are considered in the Discussion.

The subtraction of absorbance interferences from cytochrome b measurements

Measurement of cytochrome *b* kinetics commonly involves dual wavelength spectrophotometry at either 560—540 nm, or 560—570 nm. Unfortunately, neither of these wavelength pairs is entirely free of absorbance contributions from other chromatophore components [11]. In order to correct for such

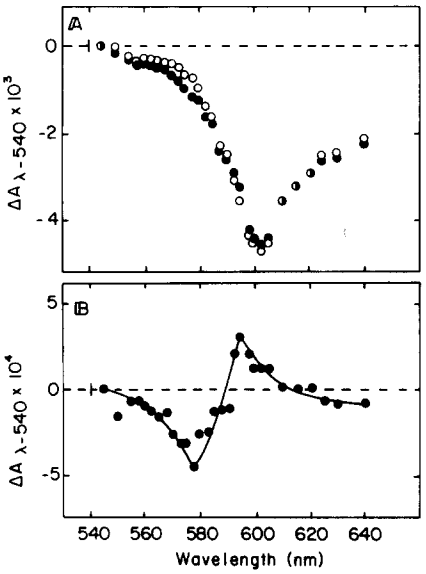

Fig. 1. Time resolved spectra of flash induced absorbance changes of $(BChl)_2^+$. Chromatophores (183 nM reaction center) in 100 mM KCl, 20 mM Mes, 1 mM $MgCl_2$, 0.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$, pH 6.0; E_h = 410 mV. A. •, ΔA 200 μs after the flash; ○, same in the presence of 5 μM valinomycin. B. Difference spectrum of the valinomycin effect.

interference, spectra of each contributing component were first obtained.

(a) Cytochrome c_2. Spectra of purified cytochrome c_2 may be found elsewhere [14]. Although the 560—570 nm wavelength pair is virtually free of contributions from cytochrome c_2 redox reactions, at 560—540 nm cytochrome c_2 oxidation would result in an absorbance increase (see Table I).

(b) The reaction center $(BChl)_2$. Fig. 1A shows the spectrum of the reaction center $(BChl)_2^+$ in chromatophores. By poising at an E_h of 410 mV such that cytochrome c_2 is oxidized prior to flash excitation, any spectral contribution from cytochrome c_2 oxidation is diminished over 10-fold. By sampling 200 μs after the flash (i.e., on a time-scale comparable to the reduction of the secondary quinone, see Refs. 15—17) the contribution from cytochrome *b* reduction is minimized. Thus, while this spectrum of the reaction center $(BChl)_2^+$ is virtually free of contributions from cytochromes *c* and *b*, it nevertheless contains a component which is recognized from its sensitivity to the addition of valinomycin (the effect of 5 μM valinomycin on the shape of this spectrum is nearly complete in 200 μs). This provides a more accurate representation of the spectrum of the chromatophore $(BChl)_2^+$, and its contribution to the 560—540 nm wavelength pair is evident; this is shown in Fig. 1A, open symbols.

(c) A red shift of the 590 nm band of antenna bacteriochlorophyll. The valinomycin sensitive part accompanying the uncorrected $(BChl)_2^+$ spectrum in Fig. 1A is shown in Fig. 1B. It appears to be a shift to the red of an absorbance maximal at 587 nm; this is probably a constituent of the antenna BChl which has an absorption maximum in the 589—590 nm region. Similar shifts identified with the antenna BChl band near 590 nm have been reported in *Rhodospirillum rubrum* [18,19] and *Rps. capsulata* [11]. The phenomenon with *Rps. sphaeroides* was first noticed by us during experiments at lower redox poten-

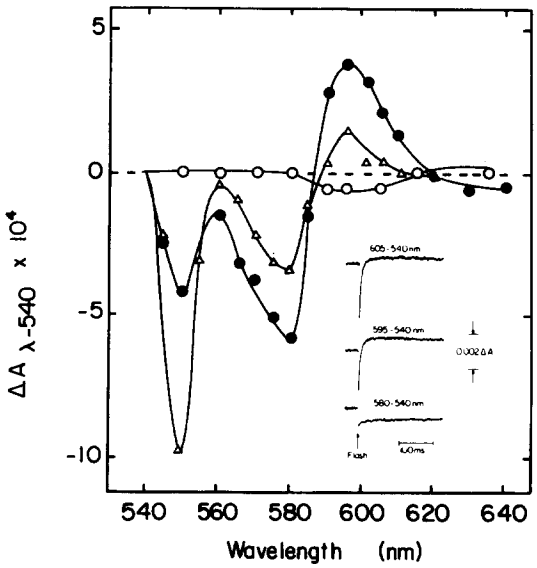

Fig. 2. Spectra of 'long lived' ΔA. Chromatophores (150 nM reaction center) in 100 mM KCl, 20 mM Mes, 1 mM $MgCl_2$, 5 μM diaminodurene, phenazine methosulfate, phenazine ethosulfate, pyocyanine and 2 hydroxy-1,4-naphthoquinone, pH 6.0; E_h = 150 mV. Absorption change 1 s after the flash is plotted: ●——●, no addition; ○——○, +3 μM FCCP; △——△, +3 μM antimycin. Inset shows the type of data from which these spectra were constructed.

tials where the shift is larger and remains for several seconds after the $(BChl)_2^+$ has been re-reduced, appearing as a net absorbance increase at 605—540 nm (see upper trace of the inset of Fig. 2). Typical spectra obtained from chromatophores poised at an E_h of 150 mV (pH 6) are shown in Fig. 2 along with examples of kinetic traces (inset) taken at different wavelengths. The figure confirms that the shift (●) is uncoupler sensitive (○) and shows that half of it is sensitive to antimycin (△). The shift assayed one second after a flash is seen without significant accompanying absorbance decrease at 605 nm, although some ferricytochrome c_2 remains oxidized and probably some cytochrome b remains reduced in the coupled state (●), and in the presence of antimycin which retards electron flow through the ubiquinone-cytochrome b/c_2 oxidoreductase. This behavior of the antenna BChl is reminiscent of red shift displayed by the carotene molecules which are also associated with the chromatophore membrane antenna proteins (see Refs. 20, 21). Further examination of this important similarity was made by comparing the flash activated kinetics of the two spectral shifts. For reasons of simplicity to avoid multiple subtraction of cytochromes in addition to $(BChl)_2$ the experiment was done at high E_h values. The antenna BChl kinetics (see Fig. 3C) were obtained by measuring at 578 nm—540 nm (Fig. 3A) which contains contributions from both the antenna BChl bandshift and $(BChl)_2^+$. The change due to $(BChl)_2^+$ alone was obtained after addition of valinomycin sufficient to collapse the bandshift to an extent greater than 95% in 200 μs (Fig. 3B); this was subtracted from the trace in Fig. 3A to yield the trace shown in Fig. 3C. There is a small stimulation of the $(BChl)_2^+$ reduction course in the tens of millisecond timescale (Fig. 3 D and E), however this is probably due to the contribution of the antenna BChl band-

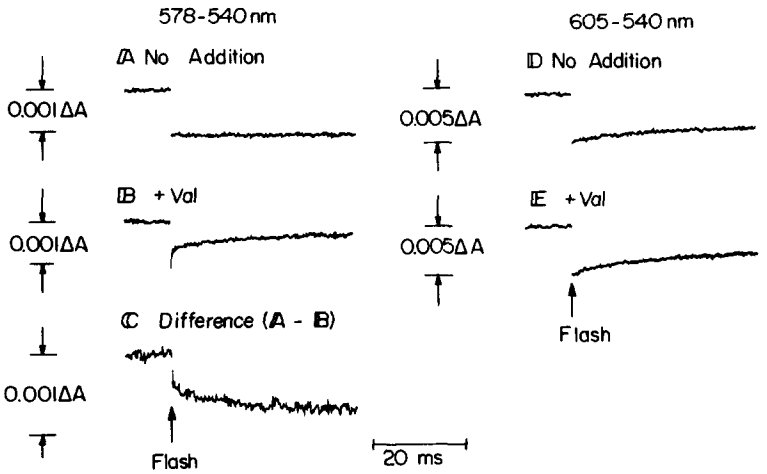

Fig. 3. Antenna bacteriochlorophyll bandshift kinetics. Conditions as in Fig. 1 except E_h = 380 mV.

shift to the $(BChl)_2^+$ absorption change at 605—540 nm (approx. 5% of the total absorption change at this wavelength pair is due to the antenna BChl shift). Fig. 4 compares the kinetic behavior and response to antimycin of the antenna BChl and carotenoid bandshifts and shows them to be remarkably similar.

Kinetic correction of cytochrome b

The corrected cytochrome *b* kinetics shown in Figs. 6—9 have had the con-

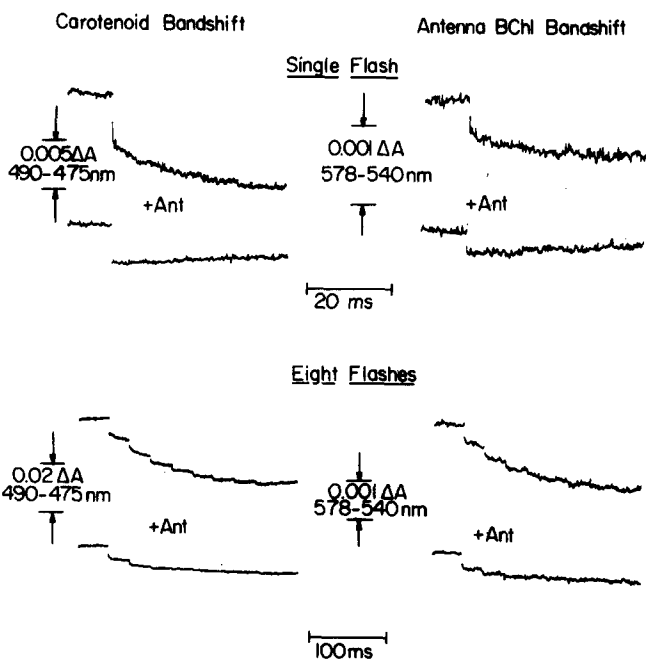

Fig. 4. Comparison of the kinetics of the carotenoid bandshift and the antenna bacteriochlorophyll bandshift. Conditions as in Fig. 1 except E_h = 380 mV, and 1 μM antimycin was added where indicated.

TABLE I

CONTRIBUTION OF COMPONENTS AT 560—540 nm

Component	Wavelengths of measurement	% Contribution at 560—540 nm
Cytochrome c_2	550—540	+15
$(BChl)_2^+$	587—540	—18
Antenna BChl bandshift	578—540 *	—21

* The actual amount of absorbance change due to this component is obtained by measuring the absorbance change in the absence of valinomycin at 578—540 nm, 45 ms after flash activation. Valinomycin (5 μM) is then added, and the absorption change is measured again (see Fig. 3). The difference between these two values is the amount due to the antenna BChl bandshift.

tributions from cytochrome c_2, $(BChl)_2^+$, and the antenna BChl bandshift removed. At each time-point shown, the contributions from the three absorbance changes interfering at 560—540 nm were subtracted. The actual amount of these contributions are shown in Table I. Oxidation of the bacteriochlorophyll dimer is measured at 587—540 nm. With this wavelength pair, there is no significant contribution from the antenna BChl bandshift to the $(BChl)_2^+$ kinetics. 587 nm is also the single beam near-isosbestic for chromatophores, so this measurement of $(BChl)_2^+$ is actually of the absorption increase at 540 nm. The amount of antenna BChl bandshift contribution in each experiment was determined from the difference brought about by 5 μM valinomycin in the flash induced kinetics at 578—540 nm: The absorption change 45 ms after a flash in the presence of valinomycin was subtracted from the absorption change 45 ms after a flash in the absence of valinomycin, yielding the net antenna BChl bandshift absorption change after 45 ms. The kinetics of the carotenoid

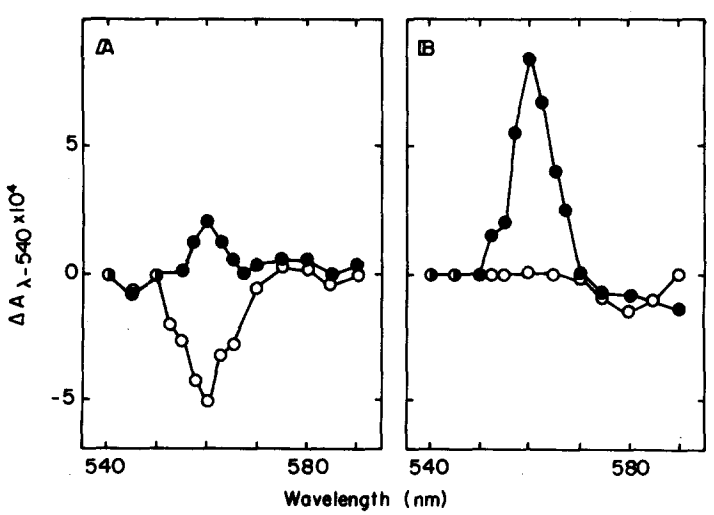

Fig. 5. Corrected difference spectrum. Contributions from cytochrome c_2, $(BChl)_2^+$, and the antenna BChl bandshift were subtracted at each wavelength. Other conditions as in Fig. 2 except 3 μM FCCP was present throughout. The absorption change was measured 45 ms following the flash. ●———●, E_h = 250 mV; ○———○, E_h = 50 mV. A. No further additions. B. +5 μM antimycin.

bandshift (following suitable calibration for amplitude) was used in the cytochrome *b* corrections because of the demonstrated kinetic similarities to the antenna BChl bandshift, and the more straightforward technique of measurement.

Cytochrome b spectra

Fig. 5 shows spectra of the absorption changes obtained following flash activation, after the preceding subtractions have been performed. The resulting spectra exhibit a single absorption band centered at 560 nm which is consistent with the identification of this change as cytochrome *b*. At E_h 50 mV there is flash activated cytochrome *b* oxidation, while at E_h 250 mV the flash causes cytochrome *b* reduction. Antimycin prevents all cytochrome *b* oxidation reactions in agreement with previous findings [6]. The spectra appear to best resemble that of cytochrome *b*-560 [2].

Results

Fig. 6 shows the data which were used to make absorption corrections of cytochrome *b* kinetic measurements made at different E_h values established before flash activation; at the bottom are shown corrected traces of flash-induced cytochrome *b* reduction and oxidation. Fig. 7 compares the corrected cytochrome *b* kinetics in the absence and presence of antimycin. From these results the following observations are made:

(a) At E_h 50 mV a flash elicits net cytochrome *b* oxidation ($t_{1/2}$ approx. 0.5 ms) which is followed by a relatively slow re-reduction ($t_{1/2}$ approx. 50 ms). The oxidation following the flash is eliminated by antimycin.

(b) At E_h 150 mV the flash now elicits a net reduction of cytochrome *b* which is followed by a prompt re-oxidation. Antimycin again eliminates the oxidation and promotes a pronounced, stable reduction in a halftime of approx. 0.5 ms.

(c) At E_h 250 mV, the prompt re-oxidation phase is not observed. Never-

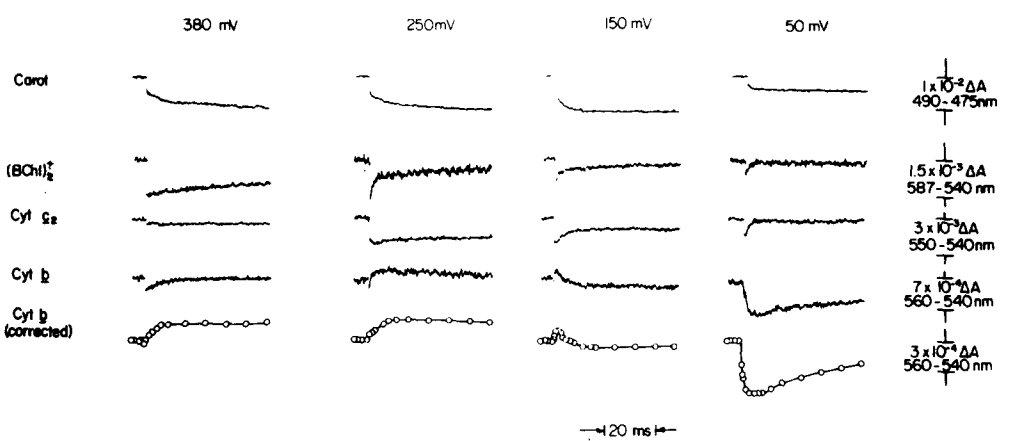

Fig. 6. Components contributing to cytochrome *b* kinetics. Conditions as in Fig. 2. Correction for cytochrome *b* kinetics is described in the text.

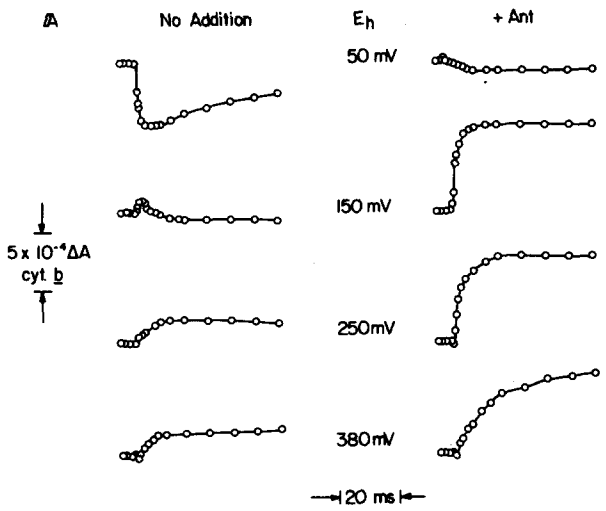

Fig. 7. Corrected cytochrome *b* kinetics. Conditions as in Fig. 2; where noted 3 μM antimycin was present.

theless antimycin promotes a 3- to 4-fold stimulation of the amount reduced. Compared with the result obtained at 150 mV the reduction kinetics may be a little slower ($t_{1/2}$ approx. 2 ms) but the amplitude is similar.

(d) At E_h 380 mV where all known redox components are oxidized with the exception of $(BChl)_2$, the results are similar to those encountered at 250 mV although in the presence of antimycin the rate of cytochrome *b* reduction is observed to be much slower ($t_{1/2}$ approx. 7 ms). The rate of ferrocytochrome *b* re-oxidation at this redox potential and at 250 mV is again very slow ($t_{1/2}$ in the hundreds of milliseconds).

These effects are shown more continuously in Fig. 8 which shows the E_h dependency of the maximum amplitude of cytochrome *b* reduced or oxidized following a flash. It is clear from Fig. 8A that in the presence of antimycin, the E_h dependency of cytochrome *b* reduction following a flash follows a simple Nernst curve with an $E_{m(6)}$ at 110 mV ($n = 1$). This is the value expected for cytochrome *b*-560 at pH 6 and this, together with the spectral data in Fig. 5 has been the basis for identifying cytochrome *b*-560 ($E_{m(7)} = 50$ mV) as the species that becomes reduced following the flash. Under these conditions there is little difference between the corrected (solid symbols) and the uncorrected (open symbols) absorbance changes. A notable aside in the figure however, is revealed at the higher E_h values where the corrected data do show a stimulatory effect of valinomycin on the amplitude of cytochrome *b* reduction.

The analysis of data obtained in the absence of antimycin (Fig. 8B) is less simple because both cytochrome *b* oxidation and reduction occur at the same time as well as to varying extents in different redox potential regions. In general the corrections are clearly more pertinent in the absence of antimycin. Included in Fig. 8B are several theoretical curves with which to compare the corrected experimental data (solid symbols). These curves show possible combinations of cytochromes *b*-560 ($E_{m(7)} = 50$ mV) and another cytochrome *b*

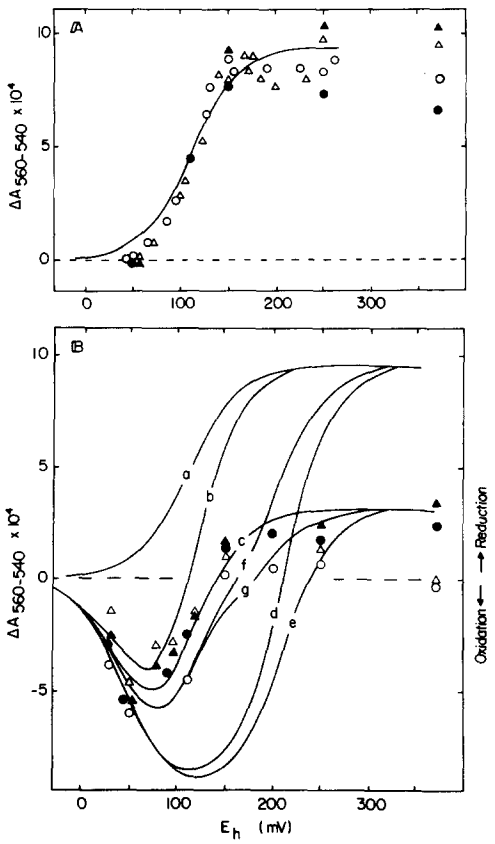

Fig. 8. Redox titration of flash induced cytochrome *b* reduction. Conditions as in Fig. 2. The absorption change plotted is the maximum change which first occurs following a flash. ●, corrected cytochrome *b* absorption change; ▲, corrected cytochrome *b* absorption change in the presence of 5 μM valinomycin; ○, uncorrected absorption change at 560—540 nm; △, same in the presence of 5 μM valinomycin. A. +2.5 μM antimycin. B. No addition: curve a, absorption change if as much cytochrome *b*-560 (50 mV) was flash reduced as in the presence of antimycin (fully reduced) and none was flash oxidized; curve b, absorption change if cytochrome *b*-560 went fully reduced if oxidized prior to the flash, and fully oxidized if reduced prior to the flash; curve c, absorption change if cytochrome *b*-560 went 33% reduced when oxidized prior to activation and fully oxidized when reduced prior to activation; curve d, cytochrome *b* ($E_{m(7)}$ = 155 mV) going fully reduced when prior oxidized and fully oxidized when prior reduced; curve e, cytochrome *b* (155 mV) going 33% reduced when prior oxidized, and fully oxidized when prior reduced; curve f, cytochrome *b* (155 mV) going fully reduced when prior oxidized, and cytochrome *b*-560 going fully oxidized when prior reduced; curve g, cytochrome *b* (155 mV) going 33% reduced when prior oxidized and cytochrome *b*-560 going fully oxidized when prior reduced.

($E_{m(7)}$ = 155 mV) and the predicted effect on the observed extent of cytochrome *b* reduction and oxidation. These possibilities are more fully explored below in the Discussion.

Discussion

Notes on the bandshifts of the antenna BChl. Comparison with carotenoid bandshifts

Although not of primary importance to this work, the following conclusions

about the spectral red shift of the antenna BChl can be made:

1. An antenna BChl absorption band maximum near 587 nm shifts to longer wavelength in response to flash-induced electron transfer steps in the reaction center and ubiquinone-cytochrome b/c_2 oxidoreductase. A similar shift in this spectral region assigned to antenna BChl has been observed also in *Rps. capsulata* following flash activation [11] and in *R. rubrum* in response to the addition of pyrophosphate [18].

2. The kinetics, the dependence of the kinetics and the amplitude of the bandshift on the redox state of the system prior to activation, and the antimycin sensitivity are very similar to those displayed by carotenoids of the chromatophore membrane [20,21]. It is accepted that the carotenoid bandshifts represent a response to electric field alterations incurred during the specific electron transfer steps in the reaction center and the ubiquinone-cytochrome b/c_2 oxidoreductase. It is probable that the antenna BChl is responding to the same events.

3. The values for the isosbestic point of the antenna BChl bandshift ranged between 585 and 587 nm, depending on the method of measurement (compare Figs. 1B and 2), and the chromatophore preparation. The peak of the absorption band of the antenna is nearer to 589 nm, so it seems possible that the red shifted antenna BChl represents a population that is already shifted several nm to the blue of the main antenna BChl peak; this is comparable to the carotenoids that undergo red shifts; these seem to originate from a carotene population (about 10% of the total [22—24]) absorbing several nm longer than the absorption peak. It is worth considering that the carotenoid and the antenna BChl may be part of the same light harvesting or antenna chlorophyll-carotenoid protein.

The quantity of cytochrome b apparent in light driven electron transport through the ubiquinone-cytochrome b/c_2 oxidoreductase

The amount of cytochrome *b* reduced following flash activation under optimal conditions in the presence of antimycin is less than 10% of the total chemically reducible material that, on the basis of absorption properties, we identify as *b*-cytochrome (data not shown). The cytochrome *b* that is flash reduced under these conditions is, from E_m and spectral information, most likely to be cytochrome *b*-560. Even comparing this flash-reducible cytochrome *b* with the total chemically reducible cytochrome *b*-560, the flash reduced extent represents little more than 20% of the total. However, if we use a reduced minus oxidized extinction coefficient for the *Rps. sphaeroides* cytochrome *b* of 10.5 $mM^{-1} \cdot cm^{-1}$ then this yields (Figs. 7 and 8A) a maximum of 0.6 cytochrome *b*-560 hemes reduced per flash activated reaction center. This extinction coefficient has been obtained (Prince, R.C., unpublished observation) from pyridine hemochromagen analysis of the isolated ubiquinone-cytochrome b/c_2 oxidoreductase which contains the full cytochrome *b* complement, not just cytochrome *b*-560 ($E_{m(7)}$ = 50 mV) so there is some room for uncertainty in the value. However, it compares reasonably well with the values determined for other *b*-cytochromes (λ_{max} 540 nm): for example 13.2 $mM^{-1} \cdot cm^{-1}$ for mitochondrial cytochrome *b* [25]; 15.9 $mM^{-1} \cdot cm^{-1}$ for chloroplast cytochrome *b*-559 [26], and 9.7 for chloroplast cytochrome *b*-563 [27].

Although the ratio of the flash reducible cytochrome *b* and reaction center is less than stoichiometric, it is nevertheless close to several other estimates of ratios of ubiquinone-cytochrome b/c_2 oxidoreductase components and reaction center. In the work of Van den Berg et al. [6] the amount of antimycin required to induce a maximal red shift in the α-band of ferrocytochrome *b*-560 as well as to inhibit several flash induced reactions in the ubiquinone-cytochrome b/c_2 oxidoreductase yielded 0.7 ± 0.1 ubiquinone-cytochrome b/c_2 oxidoreductase per reaction center (the standard deviation of ±0.1 arose predominantly not from determinations on any one preparation but from variations in chromatophores from different batches of cells). The kinetic analysis of Prince et al. [28] on the influence of reduced Q_z on the reduction rate of ferricytochrome c_2 yielded a Q_z : reaction center ratio of 0.8 ± 0.1. More recent work [4,5] also indicates that the Rieske iron sulfur cluster : reaction center ratio is approx. 0.7. Thus with the available information it is not unreasonable to conclude that there is one antimycin inhibition site, one Q_z, one Rieske iron sulfur cluster and one cytochrome *b*-560 heme functionally integral to the ubiquinone-cytochrome b/c_2 oxidoreductase, and that there are normally 0.6—0.8 ubiquinone-cytochrome b/c_2 oxidoreductases per reaction center in chromatophores from cells grown anaerobically in the light. Thus, if the above analysis of cytochrome *b* is valid we are left with a considerable amount of cytochrome *b* functionally unaccounted for.

Kinetic analysis of cytochrome b in the ubiquinone-cytochrome b/c_2 oxidoreductases

In the presence of antimycin and using near-saturating single turnover flashes the situation is simplified by the fact that cytochrome *b* undergoes prompt reduction and very much slower oxidation. The origin of the reducing equivalent involved in cytochrome *b* reduction under these inhibited conditions (cf. Ref. 6) is expected to be entirely the flash reduced Q_I of the reaction center.

The situation in the absence of antimycin is less simple because, (a) there can be both cytochrome *b* oxidation and reduction following the flash and (b) there are three distinct regimes in which quite different reactions occur following a flash. These may be summarized as follows:

1. Reduced or low redox potential conditions: cytochrome b-560, cytochrome b (155 mV), Q pool, Q_z, Rieske Fe-S, cytochrome c_2, and $(BChl)_2$ reduced; Q_I oxidized (by necessity); Q_{II} mainly reduced (see later). A single flash elicits net cytochrome *b* oxidation in $t_{1/2}$ approx. 0.5 ms. The ensuing slow re-reduction ($t_{1/2}$ approx. 50 ms) is surprising in view of the low ambient E_h and the almost fully reduced system including a flash reduced Q_I. The re-reduction halftime can be regarded only as a lower limit, since other sources of reduction (e.g., endogenous reductants; reduced redox mediators) may contribute to ferricytochrome *b*-560 reduction in this timescale. A subsequent flash delivered 24 ms later has no effect (see Fig. 9).

2. Mixed redox states or mid redox potential conditions: cytochrome b (155 mV), Q_z, Rieske Fe-S, cytochrome c_2, and $(BChl)_2$ reduced; cytochrome b-560, Q pool, Q_{II} and Q_I oxidized. A single flash elicits net reduction followed by prompt reoxidation; the whole process is essentially over in less than 10 ms. The conditions described here are optimal for the function of the reaction center-

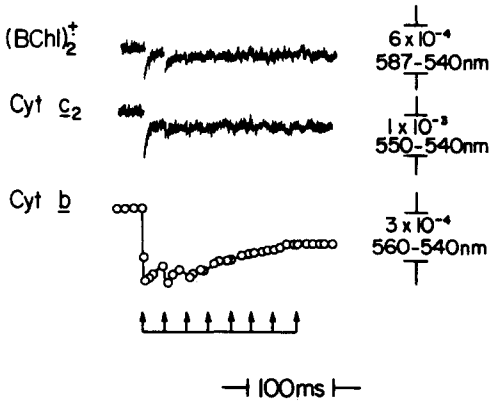

Fig. 9. 'Low E_h' multipulse kinetics. Conditions as in Fig. 2 except E_h = 50 mV.

ubiquinone-cytochrome b/c_2 oxidoreductase cycle (see Refs. 1, 3, 28 for further discussion).

3. Oxidized or high redox potential conditions: $(BChl)_2$ reduced (by necessity); cytochrome c_2, Rieske Fe-S (oxidized or reduced) cytochromes b-560, cytochrome b (155 mV) Q_z, Q_{II}, Q pool, Q_I oxidized. A single flash elicits net cytochrome *b* reduction ($t_{1/2}$ approx. 2 ms) and this is followed by slow reoxidation (hundreds of milliseconds halftime). Again the slow reoxidation is surprising because of the high ambient E_h and the highly oxidized state of the redox components of the system.

The corrected amplitudes of flash induced cytochrome *b* oxidation or reduction with (▲) and without (●) valinomycin shown in Fig. 8B are compared to several theoretical possibilities that include cytochrome *b*-560 only (curves a–c) and cytochrome *b* (155 mV) only (curves d, e), as well as combinations of both cytochromes (curves f, g). The best fit of the data is curve c; this is based solely on cytochrome *b*-560 ($E_{m(7)}$ = 50 mV) which (i) when reduced undergoes flash activated oxidation to a level that is similar to that observed maximally to become reduced in the presence of antimycin (Fig. 8A) and (ii) when oxidized undergoes flash induced reduction to a level that is about 30% of that seen in the presence of antimycin. In this analysis (see curve c) the full extent of oxidation is curtailed at low potential due to the chemical reduction of Q_I which prohibits useful photochemistry. The reason for the failure to achieve the full extent of cytochrome *b*-560 reduction at the higher potentials (i.e., following curve b) is not known for certain; we shall return to this point later. Uncorrected cytochrome *b* data qualitatively similar to curve c in Fig. 8B has been previously observed by Evans and Crofts [29] in *Rps. capsulata*. The earlier data of Dutton and Jackson [2] which was partially corrected for $(BChl)_2^+$ is similar in some respects, but notably different in the mid redox potential range (cytochrome *b*-560 oxidized, Q_z reduced).

The following schemes based on cytochrome *b*-560 alone should be considered as a model framework on which to view the oxidation-reduction kinetics of cytochrome *b*-560 in the three different regimes of starting redox states. For simplicity we have omitted in the schemes the Rieske Fe-S cluster which appears to operate between Q_zH_2 and cytochrome c_2 [4,5] and other

components we consider not immediately relevant to the discussion. In the schemes we have presented the semiquinone of Q_z and Q_{II} as $Q_z \cdot H$ and $Q_{II} \cdot H$, recognizing that other protonation states may ultimately prove more relevant.

(i) Low potential conditions. Following the flash, rapid oxidation, slow re-reduction. The oxidation of cytochrome *b* as Fig. 6 shows, appears concomitant ($t_{1/2}$ approx. 0.5 ms) with the re-reduction of flash generated ferricytochrome c_2 (see Scheme I). The brackets in this and subsequent schemes

$Q_{II} \cdot H$ or $Q_{II}H_2$	ferrocytochrome *b*	Q_zH_2	ferrocytochrome c_2	
	↓			light
$Q_{II}H_2$	ferrocytochrome *b*	Q_zH_2	ferricytochrome c_2	
	↓			step 1
$[Q_{II}H_2$	ferrocytochrome *b*	$Q_z \cdot H$	ferrocytochrome $c_2]$	
	↓			step 2
$Q_{II}H_2$	ferricytochrome *b*	Q_zH_2	ferrocytochrome c_2	

Scheme I.

indicate the presence of an unstable intermediate (see later). This pathway goes rapidly as far as the oxidation of ferrocytochrome *b*-560; it is proposed that ferricytochrome c_2 oxidizes Q_zH_2 to form $Q_z \cdot H$ which acts as an oxidant for ferrocytochrome *b*-560. The subsequent slow re-reduction of ferricytochrome *b*-560 implies that the reductive pathway, presumably via Q_I and Q_{II} to cytochrome *b*-560, is for some reason severely impeded at these low redox potentials. Indications that the impediment exists even if both Q_I and Q_{II} are reduced is shown in Fig. 9. This shows that when a train of flashes is delivered to the chromatophores poised at these low potentials, flash induced electron transfer indicated by $(BChl)_2$ or cytochrome c_2 oxidation is not evident on the third or fourth flash. This behavior is consistent with Q_I accumulating in the reduced state (preventing further useful photochemistry). We may deduce from this that any flash reduced Q_{II} is not being promptly oxidized (after each flash) in spite of the presence of ferricytochrome *b*-560.

(ii) Mid-redox potential conditions. After the flash, rapid reduction followed by rapid re-oxidation; optimal conditions for cyclic electron transfer. Two alternative schemes must be considered in this E_h region [30—32]. The first of these schemes involves reduction of ferricytochrome *b* by flash generated $Q_{II} \cdot H$ followed by oxidation of the ferrocytochrome *b* via the pathway as described in step 1 and 2 of Scheme I. However, as demonstrated by Van den Berg et al. [4], another pathway of ferricytochrome *b* reduction is possible under these conditions, shown in Scheme II. In this pathway, which conforms to

Q_{II}	ferricytochrome *b*	Q_zH_2	ferrocytochrome c_2	
	↓			light
$Q_{II} \cdot H$	ferricytochrome *b*	Q_zH_2	ferricytochrome c_2	
	↓			step 1
$Q_{II} \cdot H$	ferricytochrome *b*	$Q_z \cdot H$	ferrocytochrome c_2	
	↓			step 2
$Q_{II} \cdot H$	ferrocytochrome *b*	Q_z	ferrocytochrome c_2	
	↓			step(s) 3
Q_{II}	ferricytochrome *b*	Q_zH_2	ferrocytochrome c_2	
	↓			

Scheme II.

the Q-cycle of Mitchell [30], $Q_z \cdot H$ in step 1 acts (instead of an oxidant) as a reductant (step 2) of ferricytochrome *b*. In the Q-cycle model it is proposed in step(s) 3 that the $Q_{II} \cdot H$ acts (instead of reductant) as oxidant of cytochrome *b* yielding ferricytochrome *b* and $Q_{II}H_2$. The $Q_{II}H_2$ then reduces Q_z to Q_zH_2 [32]. The most important difference between the two pathways rests on whether the semiquinone of Q_{II} or Q_z acts as oxidant or reductant of cytochrome *b*.

(iii) High redox potential conditions. After the flash rapid partial reduction followed by slow reoxidation (Scheme III). In step 1 this scheme proceeds

Q_{II}	ferricytochrome *b*	Q_z	ferri- or ferrocytochrome c_2	
	↓			light
$Q_{II} \cdot H$	ferricytochrome *b*	Q_z	ferricytochrome c_2	
	↓			step 1
Q_{II}	ferrocytochrome *b*	Q_z	ferricytochrome c_2	

Scheme III.

rapidly as far as cytochrome *b* reduction. In this case, the slow re-oxidation of ferrocytochrome *b* has been supposed to be a result of its oxidative pathway via Q_z being impaired when Q_z is chemically oxidized prior to flash activation [33,34]. Most notable in this scheme is that although all the other components of the cycle are oxidized, the cytochrome *b* remains reduced for several hundred milliseconds. However, under these conditions (Fig. 8) the extent of reduction is only about 1/3 of that in the presence of antimycin; possible reasons for this are discussed in the next section.

The relationship of the flash activated interaction between Q_{II} and cytochrome b and their equilibrium E_m values

Rutherford and Evans [35] have recently determined in *Rps. sphaeroides* the equilibrium midpoint values for what were interpreted as the $Q_{II}/Q_{II} \cdot H$, $Q_{II} \cdot H/Q_{II}H_2$ and Q_{II}/Q_{II}^{-} couples. The $E_{m(8)}$ values found were +40 mV, −40 mV, and (pH independent) −50 mV, respectively. At our experimental pH value of 6, the E_m values for $Q_{II}/Q_{II} \cdot H$ and $Q_{II} \cdot H/Q_{II}H_2$ may be expected to be +160 and +80 mV, respectively; the $E_{m(6)}$ of cytochrome *b*-560 is between these numbers at 110 mV.

(i) Possible cytochrome b-560 Q_{II} redox interaction at high redox potentials. In the absence of antimycin there is a reasonable correspondence between the extent of cytochrome *b* stably reduced following a flash (about 30% of maximum; $K_{eq} = 0.18$) and the ΔE_m between the $Q_{II} \cdot H/Q_{II}$ and cytochrome *b*-560 couples (50 mV; $K_{eq} = 0.14$). However this correlation does not hold in the presence of antimycin where almost the full amount of cytochrome *b*-560 reduced would imply that the relevant Q_{II} couple has a significantly lower E_m relative to the cytochrome *b*-560.

(ii) Possible cytochrome b-560-Q_{II} redox interaction at low potentials. The E_m values of Q_{II} would suggest that at pH 6, $E_h = 50$ mV, Q_{II} would be 25% $Q_{II} \cdot H$ and 75% $Q_{II}H_2$ prior to activation. Yet, Figs. 6 and 9 demonstrate that reducing equivalents from $Q_{II}H_2$ are not transferred to flash generated ferri-

cytochrome *b*-560. If our interpretation of the cytochrome *b* kinetics is correct, and the equilibrium E_m values of $Q_{II}H_2/Q_{II} \cdot H$ and $Q_{II} \cdot H/Q_{II}$ are relevant to electron transfer after flash activation, we have no explanation at present based simply on redox properties for why $Q_{II}H_2$ (there before the flash or generated by the flash) does not reduce the ferricytochrome *b*-560 with a halftime much faster than the 50 ms observed.

Electron transfer pathways and energetics

The three redox regimes described in this paper each result in distinct reaction sequences following single turnover flash activation. At low redox potentials the oxidant of ferrocytochrome *b*-560 is considered to be $Q_z \cdot H$ generated by the oxidation of Q_zH_2 by ferricytochrome c_2 (via the Rieske iron-sulfur cluster). While the generation of $Q_z \cdot H$ in this way is unfavorable because the E_m of the $Q_zH_2/Q_z \cdot H$ couple is higher than those of cytochrome c_2 and the Rieske iron-sulfur cluster [6], the next step, the oxidation of ferrocytochrome *b*-560 by the $Q_zH_2/Q_z \cdot H$ couple is highly favored. Indeed there is sufficient net negative free energy change in this pair of reactions to drive the electrogenic reaction associated with the ubiquinone-cytochrome b/c_2 oxidoreductase. Consistent with this view is the similarity of the kinetics of ferrocytochrome *b*-560 oxidation, ferricytochrome c_2 reduction and carotenoid bandshift phase III and the antimycin sensitivity of all three reactions. However we do not know why there is a kinetic impediment for the presumed re-reduction via $Q_{II} \cdot H$ or $Q_{II}H_2$ of ferricytochrome *b*-560.

At high redox potentials a similar linear sequence of electron transfer steps is evident. However the oxidant for ferrocytochrome *b*-560 (reduced partially by $Q_{II} \cdot H$) is Q_z that is present there before the flash. Both the $Q_{II} \cdot H$ to ferricytochrome *b*-560 step and the subsequent ferrocytochrome *b*-560 to Q_z step are unfavorable (see Ref. 6) but the next step, the oxidation of $Q_z \cdot H$ by oxidized Rieske iron-sulfur cluster, ferricytochrome c_2 or $(BChl)_2^+$ is highly exothermic, and capable of generating an unusually slow carotenoid (or antenna BChl) bandshift phase III (note the very slow phase III at 250 and 300 mV in Fig. 7; $t_{1/2}$ approx. 25 ms). The impeded progress through the ubiquinone-cytochrome b/c_2 oxidoreductase in this case may be the two unfavorable steps that operate in tandem; more work on this matter is clearly needed.

In the mid redox potential range, where electron transfer through the ubiquinone-cytochrome b/c_2 oxidoreductase is optimal, there are several other electron transfer sequences optimal to the system. One can consider the pathway simply as a marriage of step 1 from Scheme III followed by steps 1 and 2 from Scheme I, yielding an essentially linear electron transfer sequence [32].

The Q-cycle mechanism (see Refs. 30, 31) although supported by the observation of $Q_z \cdot H$ reduction of cytochrome *b* [6], would require that ferrocytochrome *b*-560 is capable of reduction of $Q_{II} \cdot H$ to $Q_{II}H_2$. Previous assumptions concerning the $n = 2$ character of Q_{II} presented no problem for this model because the assumed high E_m of the $Q_{II} \cdot H/Q_{II}H_2$ couple (analogous to the high E_m for the $Q_z \cdot H/Q_zH_2$ couple). If $Q_{II} \cdot H$ is the oxidant functional in the Q cycle, the data of Rutherford and Evans [35] on the formation of a stable $Q_{II} \cdot H$ and the midpoint of the $Q_{II} \cdot H/Q_{II}H_2$ couple make the Q cycle model less attractive without invoking another $n = 2$ component, say Q_y,

analogous to Q_z. Ideally Q_y would be able to react with Q_I for its reduction to semiquinone, cytochrome *b* for its reduction to Q_yH_2 and with Q_z for its 2H oxidation.

Concluding remarks

In the above discussion we have not considered the involvement of the cytochromes *b* with $E_{m(7)}$ at 155 and −90 mV. Although the presence of neither is easily included in the interpretation in the experiments of the kind reported here they cannot be ruled out. In particular the experiments do not test the involvement of the cytochrome *b* with $E_{m(7)} = -90$ mV since its redox state cannot be changed prior to activation because its E_m is always lower than that of $Q_I \cdot H/Q_I$. The cytochrome *b* with $E_{m(7)} = 155$ mV on the other hand can conceivably be revealed by these experiments given appropriate kinetic behavior; however our current analyses do not reveal any compelling reason to consider it as an immediate part of the system.

Pertinent to the overall problem of electron transfer from ubiquinone to cytochrome *c* is the recent construction of a cyclic electron transfer system using isolated reaction centers, the ubiquinone-cytochrome b-c_1/c oxidoreductase from bovine heart complex III and mammalian cytochrome *b* [36]. In this simpler system we recognize the three redox regimes and in each observe similar flash activated oxidation and reduction kinetics. Thus the two redox conditions that yield apparently impeded electron flow and the narrow redox range in which rapid flow is encountered may be general. To a large degree but with some deficiencies (notably at the low potentials) we have offered reasons to explain the rates observed for each redox regime that are based solely on contraints offered by the individual chemical natures of the interacting redox centers themselves. Different conformational states with accompanying different reactivities can be invoked for each regime but until other redox based explanations have been ruled out or there is direct evidence for redox controlled conformational states this explanation remains only a possibility.

Note added in proof: (Received January 29th, 1981)

Under certain conditions, not covered in this work (after the second turnover, in the presence of antimycin) Bowyer et al. [37] have observed an absorption increase maximal at 566 nm, suggestive of photochemical reduction of cytochrome *b* (−90 mV).

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