*Biochimica et Biophysica Acta*, 723 (1983) 219–230
Elsevier



BBA 41284

# THE ROLE OF CYTOCHROME *b*-566 IN THE ELECTRON-TRANSFER CHAIN OF *RHODOPSEUDOMONAS SPHAEROIDES*

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(Received October 4th, 1982)
(Revised manuscript received January 17th, 1983)

*Key words: Bacterial photosynthesis; Cytochrome b; Electron transfer; Q-cycle; (Rps. sphaeroides)*

**Spectrophotometric, kinetic, thermodynamic and stoichiometric properties of the low-potential *b*-type cytochrome of chromatophores from *Rhodopseudomonas sphaeroides* are reported. Cytochrome *b*-566 has a double α-band with maxima at 559 and 566 nm. Resolution of the spectrum by full-spectral redox potentiometry showed no indication that the two peaks represent more than one component. The component titrated with $E_{m,7} \approx -80 \pm 10$ mV. By appropriate choice of wavelength pairs and by subtraction of the contribution due to other components, the kinetics of cytochrome *b*-566 absorbance changes following flash excitation have been resolved from those of other components. Time-resolved flash spectra corrected for the contributions of other components are consistent with the behavior of both peaks of the α-band as a single kinetic species. The kinetics of cytochrome *b*-566 in the presence of antimycin show that the reduction of this cytochrome occurred only if cytochrome *b*-561 was reduced before the flash, either chemically, by poising the ambient redox potential ($E_h$) below the $E_m$ of cytochrome *b*-561 ($E_{m,7} \approx 50$ mV), or photochemically at higher redox potentials by a previous flash. The rate of reduction of cytochrome *b*-566 varied with $E_h$. At low $E_h$ (approx. 0 mV) reduction on the first flash showed $t_{1/2} \approx 1.25$ ms; at high $E_h$ (approx. 180 mV) reduction on the second flash showed $t_{1/2} \approx 10$ ms. In the absence of antimycin at $E_h \approx 0$ mV, cytochrome *b*-566 was observed to become rapidly reduced ($t_{1/2} \approx 500$ μs) and then reoxidized ($t_{1/2} \approx 2$ ms) after a single flash. At higher redox potentials ($E_h > 80$ mV) no kinetic changes which could be unambiguously attributed to cytochrome *b*-566 were observed following a single flash. The results are interpreted in terms of a Q-cycle mechanism in which the reductant for cytochrome *b*-566 is the semiquinone formed on oxidation of ubiquinol from the quinone pool. The oxidation of the ubiquinol occurs by a concerted reaction in which one electron is accepted by the Rieske-type FeS center and the other by cytochrome *b*-566. We suggest that the kinetic characteristics may indicate a pathway for reduction of the *b*-type cytochromes in which cytochrome *b*-566 is the immediate electron acceptor and donates to cytochrome *b*-561 in a serial pathway. The experimental results in the presence of antimycin are compared with data from a computer simulation of the thermodynamic behavior of the chain, and the computer model is shown to provide an excellent fit.**

## Introduction

Chromatophores from anaerobically grown *Rhodopseudomonas sphaeroides* have been shown to contain three *b*-type cytochromes; cytochrome *b*-150 (*b*-561(150)) ($E_{m,7} \approx 150$ mV, α-peak at 561

Abbreviations: Mops, 4-morpholineethanesulfonic acid; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; Bistrispropane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

nm), cytochrome *b*-50 (*b*-561) ($E_{m,7} \simeq 50$ mV, $\alpha$-peak at 560.5 nm) [1], and cytochrome *b*-90 (*b*-566) ($E_{m,7} \simeq -90$ mV, double $\alpha$-peak at 559 and 566 nm) [1,2]. Cytochrome *b*-561 has been shown to undergo light-induced oxidation-reduction reactions following illumination both by continuous light and by single-turnover flashes in the presence [1,3,4] and absence of antimycin [1,4]. Cytochrome *b*-566 has been shown to be reduced upon continuous illumination in the presence of antimycin [3], and flash reduction of cytochrome *b*-566 was shown to occur on the second flash in the presence of antimycin [2]. No unequivocal evidence for cytochrome *b*-561(150) oxidation or reduction by single-turnover flashes has been obtained.

Recently, we have shown that the Rieske Fe-S center [5] and a bound, *c*-type cytochrome, $c_1$ [6,7], are involved in cyclic electron transport along with the previously observed components; a soluble *c*-type cytochrome (cytochrome $c_2$) [1,4], cytochrome *b*-561 [1,4,8,9], ubiquinone [10,11] and the reaction center. Because of the similarity of components present in the ubiquinol : cytochrome $c_2$ oxidoreductase (cytochrome *b*-$c_1$ complex) of the photosynthetic bacteria to those of the ubiquinol : cytochrome *c* oxidoreductase (cytochrome *b*-$c_1$ complex) of mitochondria, and in the light of our previous work [2], we set out to ascertain if the low-potential *b* cytochrome (cytochrome *b*-566), which is analogous to cytochrome *b*-566 ($E_m \simeq -40$ mV, double $\alpha$-peak at 558 and 566 nm) in mitochondria, is involved in cyclic electron transport.

In this paper we present evidence obtained through the use of time-resolved spectra of the absorbance changes following flash illumination, and through measurement of the kinetics of the cytochromes at specific wavelength pairs, to show that cytochrome *b*-566 is involved in the functioning of the cytochrome *b*-$c_1$ complex of *Rps. sphaeroides* in cyclic electron transport. We also present a thermodynamic model for the behavior of the cytochrome *b*-$c_1$ complex in the presence of antimycin which explains the results obtained here in terms of a Q-cycle mechanism [12–15].

## Materials and Methods

Chromatophores of *Rps. sphaeroides* strain Ga were prepared as described in Ref. 16. The technique of redox poising is also described in Ref. 16. Flash spectra and kinetic traces were obtained as described in Ref. 2 except that in some experiments a flow system, described below, was used to provide a fresh dark-adapted sample for each experiment. Reaction center concentrations were estimated by the method described in Ref. 2.

The flow system used here consisted of a dark, anaerobic redox vessel which could contain from 20 to 50 ml of sample. The sample was adjusted to the desired potential by small additions of concentrated sodium dithionite or potassium ferricyanide solutions. The sample was transferred anaerobically to a flow cuvette, 0.4 ml volume, through a Teflon tube surrounded by a black gas-tight tube of butyl rubber through which a stream of oxygen-free argon flowed. Transfer to the flow cuvette was driven by top pressure on the surface of the sample in the redox vessel and after flash activation of the sample, the flow cuvette was emptied back into the redox vessel, by reversal of the gas pressure difference. The system used a set of solenoid valves, which were activated under computer control, to direct the flow of the gas. This allowed a dark-adapted sample to be obtained every 15 s. The system will be described in greater detail in a subsequent paper (Robinson, H.H., and Crofts, A.R., unpublished work).

The kinetics of cytochrome *b*-566 redox changes were obtained by taking the difference in the kinetics measured at 566 and 575 nm, and then subtracting 0.5-times the cytochrome *b*-561 change, measured at 561 minus 569 nm. Traces were further corrected for the reaction center change by subtracting 0.076-times the 542 nm change.

Valinomycin and nigericin were included in kinetic experiments, as indicated in the figure legends, to allow equilibration of the proton gradient before illumination. Valinomycin also allowed rapid dissipation of the membrane potential formed by electrogenic electron transfer following flash illumination, and rapid decay of the associated electrochromic absorbance changes [2]. In some experiments valinomycin was replaced by gramicidin which, at the concentration used, more effectively eliminated the electrochromic absorbance changes.

In kinetic experiments, redox mediators of two classes were used. Substituted quinones were pre-

sent at a relatively high concentration (5–10 μM) to provide redox buffering. Reactive mediators (generally, those with relatively stable one-electron reaction intermediates) were present at a low concentration (1 μM). The concentrations of mediators were determined on the basis of extensive control experiments in which the kinetics were measured as the concentration of one mediator was varied over a wide range while the others were held constant. Extensive control experiments using oxidative or reductive titrations showed that, within experimental error (± 10 mV), redox equilibration was established reversibly at the concentrations used routinely. At these concentrations there was no significant contribution of mediator reactions to the kinetic curves measured. Control experiments showed that all absorbance changes had decayed to the dark values within a few seconds of the flash or group of flashes. In the experiments where the flow system was not used a period of 60 s or longer was allowed between groups of flashes, and prolonged dark intervals did not change the kinetics or titration curves measured.

Absorption spectra were obtained by use of a computer-linked scanning single-beam spectrophotometer described elsewhere [17]. Full-spectra redox titrations were performed as described in Ref. 18. Fitted spectra of components of known redox midpotential were obtained by a method to be fully described in a subsequent paper (Meinhardt, S.W. and Crofts, A.R., unpublished work); the method is summarized where appropriate in the text below.

## Results

*Redox titration of the low-potential cytochrome b*

We have previously reported preliminary results showing the absorption spectrum of cytochrome *b*-566 in chromatophores of *Rps. sphaeroides* at low $E_h$ [2]. Fig. 1A shows the spectrum of the

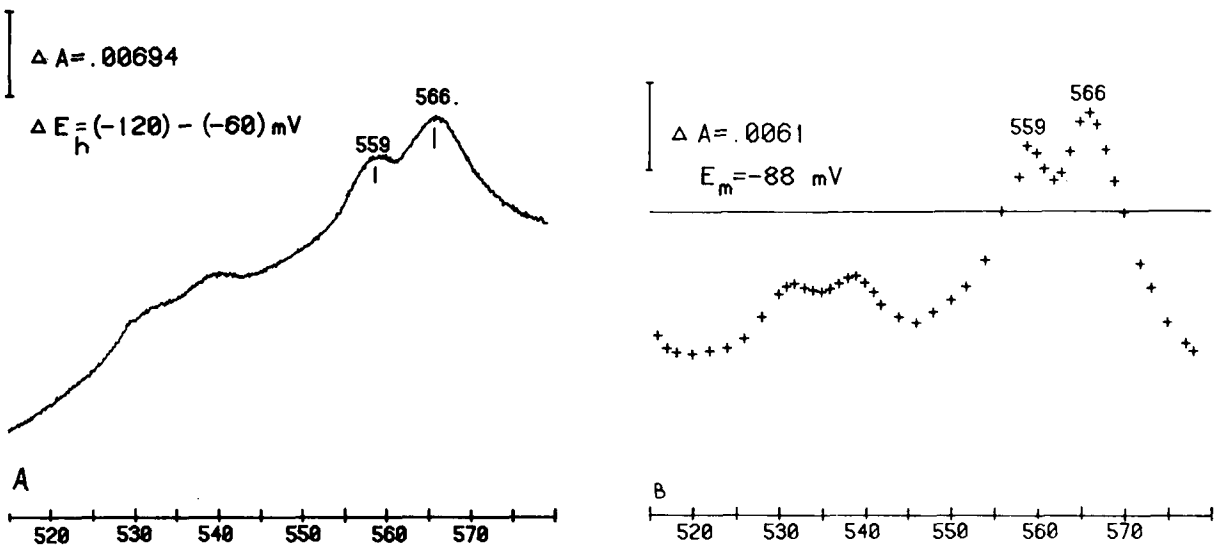


Fig. 1. Redox resolved spectra of cytochrome *b*-566. Chromatophores of *Rps. sphaeroides* Ga strain (approx. 3 μM reaction center) were suspended in 100 mM KCl, 50 mM Mops, pH 7.0, with 20 μM phenazine methosulfate, 20 μM phenazine ethosulfate, 20 μM pyocyanin; 20 μM 1,4-naphthoquinone; 20 μM 1,2-naphthoquinone; 20 μM 2-hydroxy-1,4-naphthaoquinone; 40 μM duroquinone; 100 μM DAD; 2 μM valinomycin; 2 μM nigericin. Spectra over the wavelength range 515–579 nm were measured at approx. 10 mV steps during a reductive titration over the range from 220 to − 170 mV. (A) The difference between − 120 and − 60 mV is shown. The sloping baseline is due to reduction of the mediator 2-hydroxy-1,4-naphthoquinone. (B) The absorbance change at 566–575 nm was analyzed for one, two, three or four one-electron components. The four-component fit gave $E_{m,7}$ values of 155, 50, − 88 and − 140 mV for components contributing the ratios of 1.1 : 1.3 : 1.0 : 0.1 to the change. These values were used for further analysis of the change at the other points in the α-band region; the data for the − 88 mV component are plotted here to give a spectrum. The component at − 140 mV was the mediator 2-hydroxy-1,4-naphthoquinone.

absorbance change observed at pH 7 on lowering the redox potential from −60 to −120 mV. The spectrum includes a contribution from the absorbance change due to 2-hydroxy-1,4-naphthoquinone, present as a redox mediator, which contributes a sloping baseline in the range 520–560 nm. The spectrum was obtained from a full-spectrum redox titration which showed a component of $E_m \approx -88$ mV at pH 7 on computer analysis of the absorbance change occurring at 566–575 nm. The titration curves showing changes in absorbance as a function of $E_h$ at a series of points through the spectral region from 515 to 579 nm could be extracted from a set of spectra obtained at a series of $E_h$ values on reductive or oxidative titration. By processing such titration curves, the computer could select the contribution of the component with $E_m = -88$ mV at each wavelength and plot the set of values as a function of wavelength. The resulting spectrum is shown in Fig. 1B, normalized by reference to the absorbance at 570 nm. The two spectra of Fig. 1 are clearly similar, and show a double peak in both the α- and β-bands with maxima at 566, 559, 539 and 532 nm. The lack of variation of the shape of the spectrum with $E_h$ and the similarities of the two spectra of Fig. 1 are consistent with the presence of a single component contributing the double maxima of the α- and β-bands. However, we cannot exclude the possibility that two separate components having nearly identical redox midpoint potentials contribute separate α- and β-bands to a composite spectrum as suggested for the mitochondrial cytochromes [19–22].

*Kinetics of cytochrome b-566 absorbance changes on flash illumination*

The spectra of Fig. 1, together with our previously published spectra for the absorbance changes due to oxidation of $(BChl)_2$, cytochrome $c_1$, cytochrome $c_2$ and cytochrome *b*-561 [2,23], have enabled us to select wavelengths and correction factors which allow us to measure the kinetics of cytochrome *b*-566 without major contamination from other known species (see Materials and Methods). The traces of Fig. 2 show absorbance changes due to cytochromes *b*-566 and *b*-561 following a series of flashes at several values of $E_h$ in the presence of antimycin. At $E_h$ values where

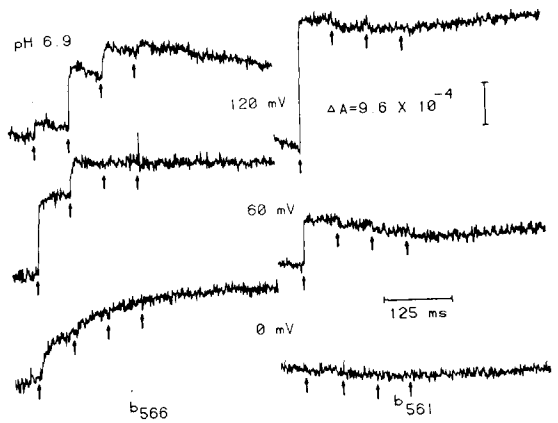


Fig. 2. Kinetics of cytochrome *b*-566 and cytochrome *b*-561 at pH 6.9. Chromatophores (approx. 0.3 μM reaction center) in 100 mM KCl, 50 mM Mops, pH 6.9, were adjusted to the $E_h$ values shown. Kinetic traces (average of four, 500 ms sweep, 500 μs filter RC, 64 ms between flashes, 60 s between groups) were accumulated at a set of wavelengths and stored for analysis. Cytochrome *b*-561 was measured at 561–569 nm; cytochrome *b*-566 was measured at (566–575 nm) − [0.5(561–569 nm)]. All changes were corrected for reaction center by subtraction of the normalized 542 nm trace. Antimycin (10 μM), valinomycin (2 μM), and nigericin (2 μM) were present for all experiments. Mediators present were as follows: 10 μM *p*-benzoquinone, 10 μM 1,2-naphthoquinone, 10 μM 1,4-naphthoquinone, 10 μM duroquinone, 2 μM DAD, 1 μM phenazine methosulfate, 1 μM phenazine ethosulfate and 1 μM pyocyanin. Flash duration was approx. 25 μs at half amplitude.

cytochrome *b*-561 was oxidized before the flash train (traces at $E_h = 120$ mV), the first flash induced reduction of cytochrome *b*-561, but little reduction of cytochrome *b*-566. On a second flash, reduction of cytochrome *b*-566 occurred with little change in cytochrome *b*-561. Subsequent flashes induced additional reduction of cytochrome *b*-566. At values of $E_h$ where cytochrome *b*-561 was partially reduced before the flash train (trace at $E_h =$ 60 mV), the first flash induced reduction of cytochrome *b*-561 (with a lesser extent) and partial reduction of cytochrome *b*-566. A second flash produced further reduction of cytochrome *b*-566 to a maximal extent. At values of $E_h$ where cytochrome *b*-561 was almost completely reduced, but the primary acceptor ubiquinone ($Q_A$) was partially oxidized before the flash train (traces at $E_h \approx 0$ mV), no marked reduction of cytochrome *b*-561 was observed, but cytochrome *b*-566 was mostly reduced on the first flash and the remain-

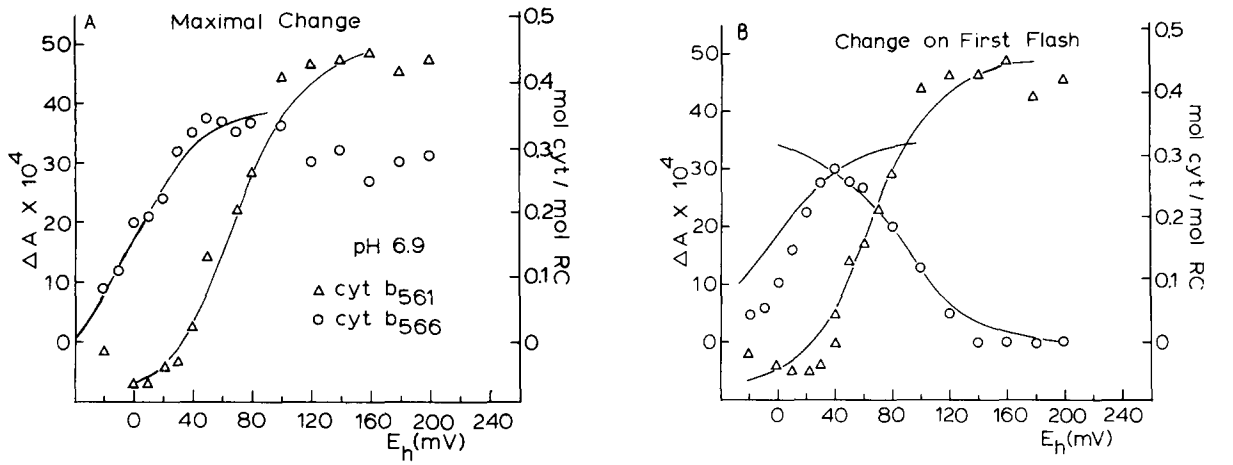


Fig. 3. Redox titration of the extent of flash-induced changes of cytochrome *b*-566 and cytochrome *b*-561 at pH 6.9. Chromatophores (approx. 0.56 $\mu$M reaction center (RC)) were suspended in the same buffer as Fig. 2 with the same mediators. Kinetic traces (average of two, 500 ms sweep, 1 ms filter RC, 64 ms between flashes, 60 s between groups) were acquired as in Fig. 2. The cytochromes were measured as described in Fig. 2. (A) The maximum change was measured as the maximum deflection of the trace from the baseline after four flashes 64 ms apart. Through the points for cytochrome *b*-561 is a hand-drawn $n = 1$ curve with $E_m = 66$ mV. Through cytochrome *b*-566 is drawn an $n = 1$ curve with $E_m = -3$ mV. (B) The change on the first flash was measured at 50 ms after the first flash. Through the points for cytochrome *b*-561 is drawn an $n = 1$ curve with $E_m = 66$ mV. Drawn through the points for cytochrome *b*-566 are two $n = 1$ curves with $E_m = 80$ and $-3$ mV. The scale on the right is based on extinction coefficients of 10.3 $mM^{-1} \cdot cm^{-1}$ at 542 nm (for the reaction center) and 19.5 $mM^{-1} \cdot cm^{-1}$ at 561 – 569 nm (for cytochrome *b*-561) (2), or at 566 – 575 nm after correction for cytochrome *b*-561 (for cytochrome *b*-566). The scale is included solely to facilitate comparison with the computer-generated curves of Fig. 6; precise values for the extinction coefficients of the *b*-type cytochromes are not known.

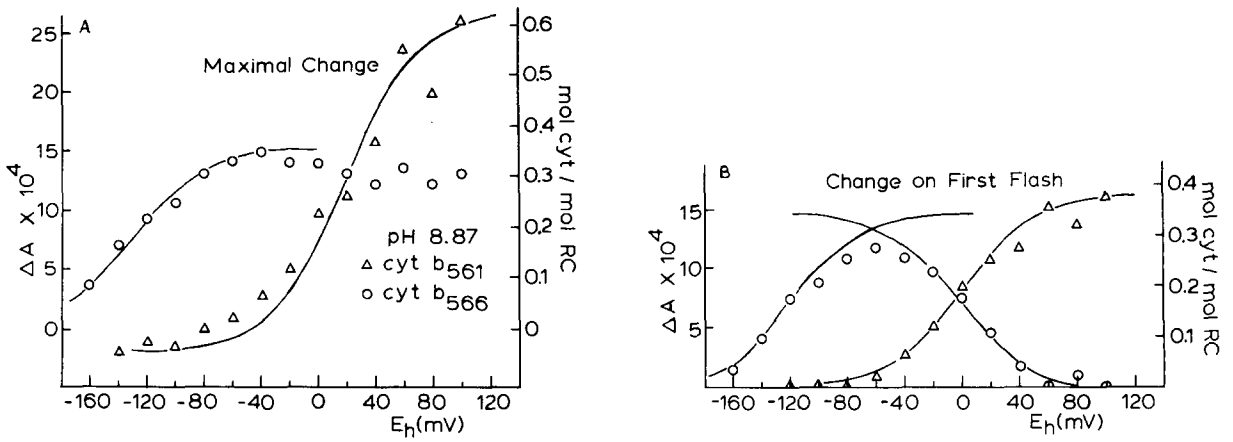


Fig. 4. Redox titration of cytochrome *b*-566 and cytochrome *b*-561 at pH 8.87. Chromatophores (approx. 0.22 $\mu$M reaction center (RC)) in 100 mM KCl, 50 mM Bistrispropane, pH 8.87, were adjusted to the $E_h$ shown. The kinetic traces (average of four sweep 200 ms, 200 $\mu$s filter RC, 32 ms between flashes, 60 s between groups) were acquired as in Fig. 2. The mediator concentrations and the measurements of the cytochromes were the same as in Fig. 2. Kinetics were measured as in Fig. 2. (A) Maximum change was measured as the maximum deflection from the baseline after four flashes 32 ms apart. Drawn through the points for cytochrome *b*-561 is a hand-drawn $n = 1$ curve with $E_m = 20$ mV. Through cytochrome *b*-566 is drawn an $n = 1$ curve with $E_m = -130$ mV. (B) The change on the first flash was measured as the change approx. 30 ms after the first flash. Through cytochrome *b*-561 is a hand-drawn $n = 1$ curve, $E_m = 0$ mV. Through cytochrome *b*-566 are drawn two $n = 1$ curves, $E_m = 0$ and $-130$ mV. The scale on the right was estimated as in Fig. 3.

ing cytochrome *b*-566 was reduced by subsequent flashes. In a similar set of experiments at pH 8.9, but performed at $E_h$ values appropriate to the midpoint potentials of the components at this pH, the kinetics of reduction of cytochrome *b*-566 as a function of flash number showed a pattern with respect to the reduction of cytochrome *b*-561 similar to that observed at pH 7. In Figs. 3B and 4B the levels of reduction of cytochromes *b*-561 and *b*-566 at a point 50 ms (Fig. 3B) or 30 ms (Fig. 4B) after one flash are plotted as a function of redox potential. The extent of reduction of cytochrome *b*-566 induced by the first flash titrated in as the $E_h$ was lowered over the range in which the flash-induced change of cytochrome *b*-561 titrates out, due to its chemical reduction before the flash. Cytochrome *b*-566 titrated out on chemical reduction of $Q_A$. The reduction of cytochrome *b*-566 on the second flash was of a constant extent over the $E_h$ range where cytochrome *b*-561 was fully oxidized before the first flash. On lowering the $E_h$, the amount of cytochrome *b*-566 reduced on the first flash increased while the amounts on the third and fourth flashes decreased to zero, after which the amount reduced on the second flash then decreased as the change on the first flash increased. The total extent of cytochrome *b*-566 reduction after four flashes was relatively constant, and the total change titrated out with the reduction of $Q_A$ as shown in Figs. 3A and 4A. The data of Fig. 5A and B are points derived from a computer model of the thermodynamic behavior of the chain in which the changes in redox levels of cytochromes *b*-561 and *b*-566 to be expected after rapid equilibration with the flash-generated redox species were calculated; these will be discussed more extensively below.

The traces of Fig. 6 show on a faster time scale the kinetics of reduction of cytochrome *b*-566 on the first and second flash at a series of potentials. The reduction had a half-time ($t_{1/2}$) of $\approx$ 1.25 ms on the first flash at $E_h$ 0 mV, the value being somewhat variable with preparation. A slightly longer half-time of 1.41 ms is seen on the second flash at $E_h$ 100 mV, where cytochrome *b*-561 was oxidized, but the ubiquinone pool was partly re-

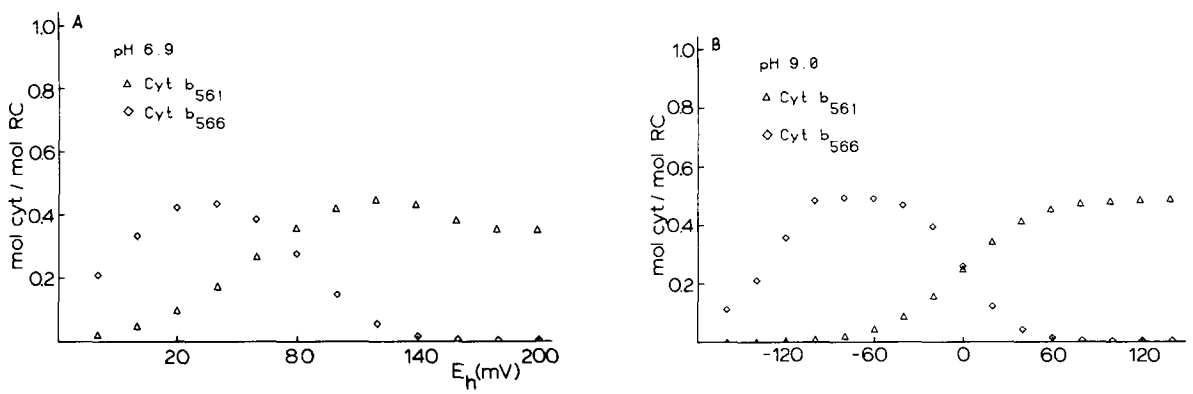


Fig. 5. Theoretical flash titrations of cytochrome *b*-561 and cytochrome *b*-566 on the first flash. The amounts of cytochrome *b*-566 and cytochrome *b*-561 reduced after one flash were calculated on the basis of a thermodynamic model of the Q-cycle. (A) At pH 6.9, the input parameters were: cytochrome *b*-561, $E_m = 56$ mV; cytochrome *b*-566, $E_m = -84$ mV; Rieske Fe-S center, $E_m = 290$ mV; cytochrome $c_1$, $E_m = 260$ mV; cytochrome $c_2$, $E_m = 345$ mV; reaction center, $E_m = 450$ mV; quinone pool, $E_m = 96$ mV; primary acceptor ($Q_A$), $E_m = 10$ mV; and saturation of reaction centers on each flash was 100%. The relative concentrations of cytochrome *b*-566, cytochrome *b*-561, Rieske Fe-S center, cytochrome $c_1$, and cytochrome $c_2$ were all 0.5 per reaction center. The concentration of $Q_A$ was 1 per reaction center and the quinone pool was made up of 30 quinones per reaction center. From each reaction center turning over, 0.5 reduced quinones were added to the quinone pool after each flash. The range of the titration was from $-20$ to 200 mV in 20-mV steps. (B) At pH 9.0 the input parameters were: cytochrome *b*-561, $E_m = 0$ mV; cytochrome *b*-566, $E_m = -210$ mV; Rieske Fe-S center, $E_m = 200$ mV; cytochrome $c_1$, $E_m = 260$ mV; cytochrome $c_2$, $E_m = 345$ mV; reaction center, $E_m = 450$ mV; quinone pool, $E_m = -30$ mV; and $Q_A$, $E_m = -110$ mV. The concentrations of all the components were as listed in A. Saturation was 90% and 0.5 reduced quinones per reaction center were added after each flash. The range of the titration was from $-160$ to 140 mV in 20-mV steps.

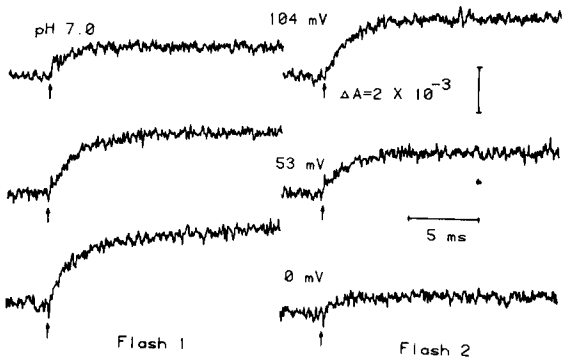


Fig. 6. Rapid kinetics of cytochrome *b*-566 following a flash in the presence of antimycin. Chromatophores (0.6 μM reaction center) were suspended in 100 mM KCl, 50 mM Mops, pH 7.0, and the $E_h$ adjusted to the value shown, using mediators as in Fig. 2. Antimycin (10 μM) and gramicidin (10 μg/ml) were also present. Kinetic traces (average of 32, 20 ms sweep, 50 μs filter RC, flashes approx. 90 ms apart) were obtained from dark-adapted samples provided by the recycling flow cuvette system described in Materials and Methods.

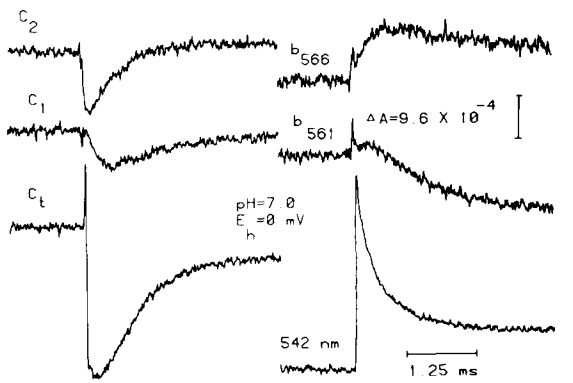


Fig. 7. Kinetics of cytochrome changes in uninhibited chromatophores. Chromatophores (approx. 0.5 μM reaction center) in 100 mM KCl, 50 mM Mops, pH 7.0, were adjusted to the $E_h$ shown. Kinetic traces (average of 32, 5 ms sweep, 10 μs filter RC) were obtained from dark-adapted samples from a recycling flow cuvette system. Cytochrome $c_t$ (total cytochrome *c*) was measured at 551 nm, cytochrome $c_1$ at 552–548 nm, and cytochrome $c_2$ at 550–554 nm. Cytochrome *b*-561 and cytochrome *b*-566 were measured as described in Fig. 2. All traces were corrected for the reaction center change by subtraction of the normalized 542 nm trace. The mediators present were 1 μM phenazine methosulfate, 1 μM phenazine ethosulfate, 1 μM pyocyanin, 5 μM duroquinone, 5 μM 1,2-naphthoquinone, 5 μM 1,4-naphthoquinone, and 5 μM 2-hydroxy-1,4-naphthoquinone. Also present were 2 μM valinomycin and 10 μg/ml gramicidin.

duced before the flash, while at values of $E_h > 200$ mV, the half-time after a second flash was much slower ($t_{1/2} \approx 7$–10 ms). Because of the subtractions required to correct these traces, the noise level, especially shortly after the flash where rapid transient changes due to the reaction center have cancelled, makes the measurement of lags in the traces somewhat ambiguous.

In Fig. 7 the kinetics of absorbance changes of the easily measured components of the chain are shown following flash activation in the absence of inhibitors, at $E_{h,7} = 0$ mV, where cytochrome *b*-561 was reduced before the flash. The traces show a rapid reduction ($t_{1/2} \approx 500$ μs) of cytochrome *b*-566 followed by a slower oxidation ($t_{1/2} \approx 2$ ms), and a slow oxidation of cytochrome *b*-561 ($t_{1/2} \approx 2$ ms), which occurs after a lag of approx. 500 μs during which cytochrome *b*-566 was being reduced. After oxidation (kinetics unresolved on this time scale), reduction of most of the cytochrome $c_2$ occurs rapidly after the flash ($t_{1/2} \approx 300$ μs), oxidation of cytochrome $c_1$ occurs with a $t_{1/2} \approx 200$ μs, followed by reduction with kinetics similar to that of the oxidation of the *b*-type cytochromes ($t_{1/2} \approx 2$ ms).

*Time resolved spectra of the flash-induced changes*

In the kinetic traces of Figs. 2–7 above, the wavelength pair 566–575 nm was used to follow the changes due to cytochrome *b*-566. This pair of wavelengths would not have included any large contribution from the peak at 559 nm, if this had been due to a separate component. We have previously shown that the spectrum of the component reduced on a second flash in the presence of antimycin at $E_h \approx 190$ mV was similar to the redox difference spectrum of cytochrome *b*-566, showing the characteristic double α-band [6]. In Fig. 8 we show time-resolved spectra of the changes following flash illumination under the other conditions in which reduction of cytochrome *b*-566 is observed. In each case, the spectra were corrected by subtraction of the changes due to $(BChl)_2$ oxidation, and, when necessary, for the change due to cytochromes $c_1$ and $c_2$ as measured at 551–542 nm. (The latter correction does not distinguish between the separate absorbance changes of the two *c*-type cytochromes, and the difference between the spectra results in the appearance of a

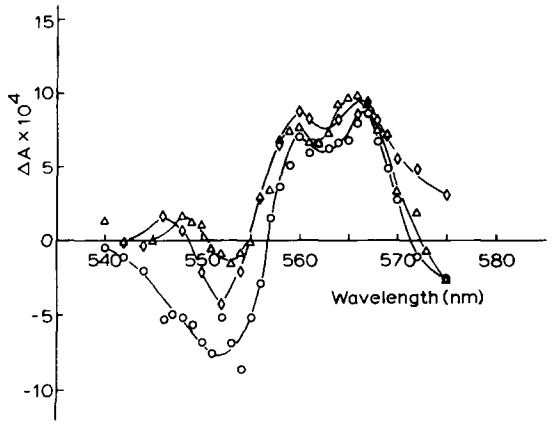


Fig. 8. Time-resolved spectra of the flash-induced changes of cytochrome *b*-566 under several different conditions. (△———△) Chromatophores were suspended to approx. 0.5 μM reaction center in 50 mM Bistrispropane, 100 mM KCl, pH 8.95, with the following mediators: 2 μM phenazine methosulfate, 2 μM phenazine ethosulfate, 2 μM pyocyanin, 10 μM 1,2-naphthoquinone, 10 μM 1,4-naphthoquinone, and 10 μM duroquinone. Also present were 10 μM antimycin, 2 μM valinocycin and 2 μM nigericin. Kinetic traces (average of 16, 20 ms sweep, 20 μs filter RC) were obtained from a dark-adapted sample supplied by a recycling flow cuvette system with 50 ml of sampled poised at $-42 \pm 3$ mV. Shown are the changes from 0.2 to 1.0 ms after the flash. Changes are corrected for the reaction center change. (○———○) Chromatophores (approx. 0.5 μM reaction center) were suspended in 100 mM KCl, 50 mM Mops, pH 7.0, with the following mediators: 1 μM phenazine ethosulfate, 1 μM pyocyanin, 10 μM duroquinone, 1 μM nigericin, 4 μg/ml gramicidin and 10 μM antimycin. The $E_h$ was adjusted to $0 \pm 5$ mV and traces (average of 16, 5 ms sweep, 10 μs filter RC, 60 s between flashes) were obtained as in Fig. 2. The spectra show the changes that occurred from 20 μs to 1.22 ms after the flash and are corrected for changes due to the reaction center, and cytochrome ($c_1 + c_2$). (◇———◇) Conditions were the same as in Fig. 7. The spectra show the changes which occurred over the interval from 140 to 390 μs after the flash and are corrected for changes due to the reaction center and cytochrome ($c_1 + c_2$).

spectrum corresponding to reduction of cytochrome $c_2$ and oxidation of cytochrome $c_1$ for points in the 'corrected' spectra in the time range 50 μs–1 ms.) For each of the experimental conditions under which reduction of cytochrome *b*-566 was shown to occur, the time-resolved difference spectrum over the time scale of reduction shows a component with a double α-band of similar shape and with the same values for $\lambda_{max}$ as that of the redox-difference spectra of Fig. 1. There is no indication in these results of any kinetic inhomogeneity in the behavior of the two peaks of the α-band spectrum. Although we cannot exclude the possibility that two components showing very similar kinetic behavior contribute to composite time-resolved spectra, we can say on the basis of these results and those of Fig. 1 that if two components do contribute to the spectrum, they behave for kinetic and thermodynamic purposes as one. The assignment of the double α-band of cytochrome *b*-566 to a single heme is supported by a magnetic circular dichroism (MCD) study of the low-potential *b* cytochrome of the mitochondrial cytochrome $b$-$c_1$ complex ($E_{m,7} \approx -40$ mV, double α-band, $\lambda_{max} = 558$ and 566 nm); it was concluded that the MCD spectra were best explained by a single heme with a split double α-band [24].

## Discussion

*Reduction of cytochrome b-566*

The redox midpoint potential of cytochrome *b*-566 has a value of −90 mV at pH 7 and varies with pH by approx. −59 mV/pH unit [9]. No $pK_a$ value has yet been measured. It seems likely that cytochrome *b*-566 is reduced as an H carrier with the same operating $E_m$ as that measured in equilibrium redox titration, since the $E_m$ value of the electron-carrying couple would be lower than −210 mV, which is below the operating potential of the primary quinone acceptor. Even with a value for $E_{m,7}$ of −90 mV, problems arise in considering candidates for the reductant for cytochrome *b*-566. Flash-induced reduction occurs at values of $E_h$ below that at which the secondary acceptor titrates out ($E_{m,7} \approx 40$ mV), as measured by the turnover of the reaction center on a second flash following shortly (500 μs) after the first (Cogdell, R.J., Jones, K.R. and Crofts, A.R., unpublished observations). It is clear that at a value for $E_{h,7}$ of approx. 0 mV, an electron leaves $Q_A^-$ in only a small fraction of the centers activated by a first flash, but nevertheless, a substantially full reduction of cytochrome *b*-566 occurs (Figs. 2 and 7). We may therefore exclude $Q_A^-$ as the reductant for cytochrome *b*-566. It also seems unlikely for thermodynamic reasons that either of the couples of the secondary quinone acceptor acts as reductant. The $E_{m,7}$ values for the protonated species measured by Rutherford and Evans [25] are both

much higher than that of cytochrome *b*-566, and the only couple likely to provide sufficient reducing potential would be $QH^{\cdot}/QH^{-}$ [25], which would not be expected to be present at a significant concentration under equilibrium conditions because of the high $pK_a$ for dissociation of the quinol. Furthermore, oxidation of $Q_B$ in any of its reduced forms would provide an acceptor for electrons from $Q_A^{-}$, and no oxidation of $Q_A^{-}$ was observed over a time scale of several milliseconds. No other stable component of the chain has a value for $E_{m,7}$ low enough to provide sufficient reducing potential.

Two different sorts of mechanisms might be envisaged to account for the reduction of cytochrome *b*-566. We have previously suggested the possibility that the operating $E_m$ for the cytochrome might be modified by interaction with the oxidized form of one of the high-potential components, so as to raise its value to a range in which cytochrome *b*-566 could act as acceptor from one of the low-potential components (the couple $Q_B^{\cdot}H/Q_BH_2$, for example) [6]. We recognized that this suggestion, though not unreasonable, involved a number of ad hoc postulates for which there was no experimental justification, and we now prefer the alternative explanation below. The second possibility is that a reductant of low redox potential is produced dynamically, as in the oxidation of ubiquinol to an unstable semiquinone as originally suggested by Wikström and Bearden [26], and incorporated by Mitchell [12,13] into the Q-cycle mechanism. We have recently discussed a variant of the Q-cycle which explains with great economy many of the kinetic phenomena which were previously anomalous, and we will discuss our present result in the context of this new formulation [27].

*Interpretation of the role of cytochrome b-566 in terms of a Q-cycle*

We have presented a summary of the postulates leading to our new formulation of the Q-cycle mechanisms elsewhere [27], and have discussed these and their justification at greater length in the preceding paper [28] which contains a scheme showing the components of the cyclic chain of *Rps. sphaeroides* arranged as a Q-cycle, and a table summarizing pertinent thermodynamic, kinetic and stoichiometric parameters for the components and reactions of the cycle. Points of importance in the context of the present discussion are outlined below.

(a) The ubiquinol : cytochrome $c_2$ oxidoreductase complex acts to oxidize ubiquinol which has the thermodynamic and kinetic characteristics of the quinone pool. This point is justified and discussed at length in the accompanying paper [28].

(b) Ubiquinol from the pool is oxidized by a second-order reaction with the complex, described by the equation:

$$QH_2 + (FeS^{+} \cdot b\text{-}566) \rightleftharpoons Q + (FeS \cdot b\text{-}566H) + H^{+} \quad (1)$$

The physiological equilibrium constant is given by:

$$K_{eq} = \exp\left[\{(E_m(FeS) + E_m(b\text{-}566)) - 2E_m(Q)\}F/RT\right] \quad (2)$$

and has the value of approx. 2.20 at pH 7, 25°C.

(c) Following a flash, cytochrome $c_1$, cytochrome $c_2$, the FeS center and $(BChl)_2$ come to redox equilibrium. Cytochromes *b*-566 and *b*-561 also come to redox equilibrium with each other at a rapid rate (which is not rate determining for the overall process), but do not equilibrate with the high-potential components. Effectively, the disproportionation reaction at the quinol-oxidizing site does not occur at a rate comparable with the turnover of the chain.

(d) In the presence of antimycin the oxidation of the *b*-type cytochromes is inhibited, and the reaction of Eqn. 1 comes rapidly to a quasi equilibrium. Given that the high- and low-potential chains do not interact (see c, above), the concentration of the reactants of Eqn. 1 after equilibration can be established from the relationships:

$$2E'_Q = E'(FeS) + E'(b\text{-}566) \quad \text{and} \quad E'(FeS) = E'(b\text{-}566) \quad (3)$$

where $E'$ is the effective potential of the redox couple indicated, given by:

$$E' = E_m + \frac{RT}{zF} \ln \frac{(\text{oxidized})}{(\text{reduced})}$$

where $R$, $T$ and $F$ have their conventional meanings, and $z$ is the electron stoichiometry for the couple.

Using these postulates and those discussed in

detail in the accompanying paper [28] as a framework, we have written a computer program to calculate the changes in concentration of the components of the chain following one or two flashes in the presence of antimycin. Input parameters are the stoichiometries and redox midpoint potentials of all the components; the initial value for $E_h$ (the ambient redox potential with which the components equilibrate before the flash), the value by which this is to be incremented for each cycle of the program, and the finishing $E_h$ value; the saturation of the flash; and the fraction of centers containing $Q_B^-$ before the flash. The percent saturation of the flash, and the stoichiometry of reaction centers with respect to the chain determine how many electrons are abstracted from the high-potential end of the chain, and these factors, together with the fraction of centers with $Q_B^-$, determine the number of equivalents of $QH_2$ introduced to the quinone pool. The program then uses an iterative procedure to compute the distribution of oxidizing and reducing equivalents which satisfies the relationship of Eqns. 2 and 3 and postulate c.

In the context of the assumptions above, the following series of events account for the results observed.

*(1) At $E_{h,7} \approx 200$ mV.* At this potential the ubiquinone pool, cytochrome *b*-561 and cytochrome *b*-566 are oxidized, and the high-potential components (FeS center, cytochrome $c_1$, cytochrome $c_2$ and $(BChl)_2$) are all reduced before the flash.

(a) Excitation leads to oxidation of $(BChl)_2$ (to give approx. 2 $(BChl)_2^+$/complex), reduction of Q to $Q_B^-$ in approx. 50% of the centers, and reduction of $Q_B^-$ to $QH_2$ in the remaining centers, to give approx. 1 $QH_2$/complex in the pool.

(b) $(BChl)_2^+$ is reduced by electrons from the high-potential chain leading after a brief lag (100–200 $\mu$s), to oxidation of the FeS center, with $t_{1/2} \approx 200$–$300$ $\mu$s.

(c) $QH_2$ produced at the $Q_B$ site diffuses via the pool to the $Q_Z$ site.

(d) The reaction of Eqn. 1 occurs.

(e) The electron passed to the FeS center is shared among the high-potential components, according to their relative $E_m$ values.

(f) Cytochrome *b*-566 reduced by the reaction is rapidly oxidized by cytochrome *b*-561, so that no significant increase in concentration of reduced cytochrome *b*-566 occurs.

(g) In the presence of antimycin, cytochrome *b*-561 becomes stoichiometrically reduced, and remains reduced. The rate and stoichiometry of reduction are determined by the second-order reaction with $QH_2$ from the pool.

(h) Cytochrome *b*-566 remains oxidized after transient reduction, because all the reductant has been consumed.

(i) Following a second flash, the sequence a–e is repeated, but now the electron remains on cytochrome *b*-566, because its oxidant, cytochrome *b*-561, is already reduced by the preceding flash.

(j) In the absence of antimycin the sequence following the first flash is the same through to f, but the cytochrome *b*-561 reduced is reoxidized by ubiquinone from the pool at a separate catalytic site (the antimycin-binding site) in an electrogenic reaction, producing $QH_2$ which recycles over a longer time scale around the sequence of reactions outlined above [28].

*(2) At $E_{h,7} \approx 100$ mV.* At this potential the ubiquinone pool is approx. 30% reduced, the high-potential components are completely reduced and the *b* cytochromes are largely oxidized.

(a) The sequence of reactions above proceeds, except that now the rate of the reaction of Eqn. 1 is no longer limited by diffusion of $QH_2$ from the $Q_B$ site [28].

(b) In the presence of antimycin, following one flash, the reaction of Eqn. 1 proceeds to equilibrium, as defined above (Eqns. 2 and 3). Although $QH_2$ is present in excess, the extent of reduction of cytochrome *b*-566 is limited by the degree to which the high-potential chain and cytochrome *b*-561 have become reduced on oxidation of the first equivalent to $QH_2$ (see below). The data of Fig. 5 show the extent of reduction of the two *b*-type cytochromes to be expected after a single flash at the $E_h$ values indicated, given the assumptions summarized in the model above and in the figure legends.

(c) Following a second flash the reaction of Eqn. 1 is pulled over to the right by oxidation of the FeS center, and cytochrome *b*-566 reduction occurs as equilibrium is reestablished.

*(3) At $E_{h,7} \approx 0$ mV.* At this potential the primary

acceptor, $Q_A$, and cytochrome *b*-561 are, respectively, 40–50 and 85–90% reduced, the quinone pool and the high-potential components are practically completely reduced, and cytochrome *b*-566 is 96% oxidized.

(a) In reaction centers in which $Q_A$ was oxidized before the flash, $(BChl)_2^+$ and $Q_A^-$ are generated, and the reactions on the donor side proceed as above, leading to oxidation of the FeS center. On the acceptor side, $Q_A^-$ remains reduced on the millisecond time scale.

(b) The reaction of Eqn. 1 occurs, leading to the reduction of cytochrome *b*-566 and of the FeS center (leading to loss of one oxidizing equivalent in the high-potential chain) and the generation of 1 Q/complex in the pool.

(c) In the absence of antimycin, the quinone diffuses to the quinone reductase site, and oxidizes both cytochrome *b*-561 and cytochrome *b*-566.

(d) On oxidation of cytochrome *b*-566, the quinol oxidase site (the reaction of Eqn. 1) can turn over a second time, leading to reduction of the components of the high-potential chain (mainly cytochrome $c_1$) which remained oxidized after the first turnover of the site, and rereduction of cytochrome *b*-561. The quinone produced is available for reduction either at the reductase site of the complex, or by $Q_A^-$ of the reaction center.

The sequence of events discussed above provides a satisfactory explanation for the kinetic and thermodynamic behavior of the *b*-type cytochromes in terms of a Q-cycle in which the cytochromes are arranged in series. Such a serial arrangement is strongly indicated by the differential effects of antimycin and myxothiazol on cytochrome *b*-561 and cytochrome *b*-566, respectively [30]. On the basis of our present results, an alternative plausible arrangement would be one in which the *b*-type cytochromes were arranged in parallel as alternative acceptors for the low-potential electron from the quinol oxidase site as suggested by Velthuys [31]. In this case it would be necessary to postulate that the two were in rapid equilibrium so that the distribution of electrons would be determined solely by the relative electron affinities ($E_m$ values). A more general scheme would have the *b* cytochromes available as a two-electron storage device, without specifying the pathway. In any case, it is clear from the sensitivity to antimycin that the electrogenic event indicated by the slow phase of the carotenoid change is not associated with reduction of the *b* cytochromes, but with their oxidation.

In the above discussion we have assumed that the complex functions as a monomeric unit. In mitochondria, there is a considerable body of evidence to suggest that the ubiquinol : cytochrome *c* oxidoreductase complex occurs and functions as a dimer [32]. The mechanism we have suggested can be readily adapted to this situation by postulating that the quinone reductase site is formed at the dimer interface, enabling the dimeric complex to reduce quinone by a single electron transfer from each half of the dimer. In this case, a single turnover of the dimeric complex would lead to oxidation of two equivalents of quinol and reduction of one equivalent of quinone, to give a net oxidation of 1 quinol/dimeric complex.

## Acknowledgements

We would like to thank Ms. Lee Ann Oimoen and Ms. Kally Webster for their expert technical assistance, and Mr. Alex Lam for his electronics assistance. This research was supported by a grant from the National Institute of Health PHS 5 RO1 GM26305.

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