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SINGLE AND MULTIPLE TURNOVER REACTIONS IN THE UBIQUINONE-CYTOCHROME *b*-c_2 OXIDOREDUCTASE OF *RHODOPSEUDOMONAS SPHAEROIDES*

THE PHYSICAL CHEMISTRY OF THE MAJOR ELECTRON DONOR TO CYTOCHROME c_2, AND ITS COUPLED REACTIONS

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SUMMARY

We have examined the thermodynamic properties of the physiological electron donor to ferricytochrome c_2 in chromatophores from the photosynthetic bacterium *Rhodopseudomonas sphaeroides*. This donor (Z), which is capable of reducing the ferricytochrome with a halftime of 1–2 ms under optimal conditions, has an oxidation-reduction midpoint potential of close to 150 mV at pH 7.0, and apparently requires two electrons and two protons for its equilibrium reduction.

The state of reduction of Z, which may be a quinone · protein complex near the inner (cytochrome c_2) side of the membrane, appears to govern the rate at which the cyclic photosynthetic electron transport system can operate. If Z is oxidized prior to the flash-oxidation of cytochrome c_2, the re-reduction of the cytochrome takes hundreds of milliseconds and no third phase of the carotenoid bandshift occurs. In contrast if Z is reduced before flash activation, the cytochrome is rereduced within milliseconds and the third phase of the carotenoid bandshift occurs. The prior reduction of Z also has a dramatic effect on the uncoupler sensitivity of the rate of electron flow; if it is oxidized prior to activation, uncoupler can stimulate the cytochrome re-reduction after several turnovers by less than tenfold, but if it is reduced prior to activation, the stimulation after several turnovers can be as dramatic as a thousandfold. The results suggest that Z plays a central role in controlling electron and proton movements in the ubiquinone cytochrome *b*-c_2 oxido-reductase.

INTRODUCTION

The photosynthetic energy conserving chromatophore membranes of *Rhodospeudomonas sphaeroides* contain the reaction center protein which converts light energy into electrochemical potential free-energy. The reaction center protein is located within the chromatophore membrane matrix, and serves to drive a cyclic electron flux, which is apparently also coupled to proton movement, through a ubiquinone-cytochrome b-c_2 oxidoreductase. If excited by a quantum of light, the reaction center bacteriochlorophyll [a bacteriochlorophyll dimer, designated $(\mathrm{BChl})_2$] undergoes oxidation [to $(\mathrm{BChl})_2^{\dot{+}}$] and delivers one electron via an intermediary carrier ($\mathrm{I}^{\dot{-}}/\mathrm{I}$), to a ubiquinone · iron complex (the reaction center "primary acceptor", designated $\mathrm{Q}^{\dot{-}}\mathrm{Fe}/\mathrm{QFe}$) nearer the outside of the membrane (for a recent review on electron transfer within the reaction center, see ref. 1). The $\mathrm{Q}^{\dot{-}}\mathrm{Fe}$ transfers one electron out of the reaction center to a ubiquinone (Q) complement, which binds a proton from the external aqueous phase forming $\mathrm{Q}^{\cdot}\mathrm{H}$ in 150 μs at pH 7 [2]. Meanwhile, the $(\mathrm{BChl})_2^{\dot{+}}$ formed in the light reaction serves to oxidize a single cytochrome c_2 heme at the inner membrane-aqueous interface [3]. There are two cytochrome c_2 molecules per reaction center [4, 5], and the oxidation of each is biphasic, each phase being approximately equal in extent and having half times of 20 and 300 μs (4). Thus, well within a millisecond following a single turnover of the reaction center, one reducing equivalent and proton enters the Q complement near the outside of the membrane and one oxidizing equivalent is created in cytochrome c_2 on the inner membrane aqueous interface. The electron returns from the reduced quinone to ferri-cytochrome c_2 via the Q-cytochrome b-c_2 oxido-reductase, and the free-energy made available during this process contributes to drive the phosphorylation of ADP. However, details of the steps taken by electrons and protons in the Q-cytochrome b-c_2 oxido-reductase remain fragmentary, as indeed is our understanding of the energy coupling mechanism.

This report is directed to determining the electrochemical properties of the immediate electron donor(s) to oxidized cytochrome c_2, and also to find out how this electron transfer reaction controls the operation of the Q-cytochrome b-c_2 oxido-reductase and the energy coupling process. A variety of candidates for the electron donor to cytochrome c_2 [E_m (pH independent) 295 mV, refs. 4, 5] are known to be associated with the chromatophore membrane, including (a) cytochrome b_{-90} (b-564) [reduced α band at 564 nm, E_m (pH 7.0) −90 mV, ref. 6]; (b) cytochrome b_{50} (b-560) [reduced α band at 546 nm, E_m (pH 7.0) 50 mV, refs. 6, 7]; (c) cytochrome b_{155} (b-558) [reduced α band at about 558–560 nm, E_m (pH 7.0) 155 mV, ref. 6]; (d) the Rieske iron sulfur protein [EPR absorbance at g 1.90 in the reduced form, E_m (pH 7.0) 285 mV, ref. 8]; (e) an iron sulfur protein, characterized by EPR absorbance at g 1.94 in the reduced form [E_m (pH 7.0) +40 mV, ref. 8] and (f) ubiquinone [9, 10], the electrochemistry of which is not known with any certainty, although in mitochondria an E_m value ($n = 2$) of 65 mV (pH 7.0) has been determined for at least some of the Q complement [11]. In addition, a hypothetical carrier, "Z" [E_m (pH 7.0) 120 mV], has been championed for some time by Crofts and coworkers (e.g. 12, 13). It is noteworthy that a quite similar choice is still available in the mitochondrial Q-cytochrome b-c_1 oxido-reductase.

Current information on electron transfer to the flash-generated ferri-cytochrome c_2 can be summarized as follows: No evidence has yet been obtained for electron donation from cytochrome b_{-90} (b-564), the g 1.94 [E_m (pH 7.0) 40 mV] iron sulfur center or the Rieske iron sulfur center, to the oxidized cytochrome c_2. A major contender as a direct donor is cytochrome b_{50} (b-560), since its oxidation kinetics ($t_{\frac{1}{2}}$ 1.7 ms) have been matched with cytochrome c_2 reduction [14], although this matching was only demonstrated under uncoupled conditions with multiple single turnover activation. The hypothetical intermediate Z has been proposed to act between cytochromes b_{50} and c_2 [12, 13], while another possibility which has been considered is that cytochrome b_{155} (b-558) may be capable of reducing ferricytochrome c_2, although not as a member of the energy-linked reaction center-Q-cytochrome b-c_2 oxidoreductase system, and only following the first flash [6]. Irrespective of the donor to cytochrome c_2 or the coupling status of the membrane, antimycin acts to drastically inhibit all electron transfer to flash generated ferricytochrome c_2 after the first and subsequent flash activations [14].

Electron transfer in the Q-cytochrome b-c_2 redox region also appears to be coupled to a red shift in the spectrum of the carotenoid complement of the chromatophore membrane. This red shift (designated phase III) has a halftime of formation in the 1–2 ms time range, and is inhibited by antimycin [15]. The origin of two other faster and antimycin insensitive carotenoid bandshifts seems reasonably clear; they appear to be coupled to electron transfer from $(BChl)_2$ via I to QFe (Phase I) and from ferro-cytochrome c_2 to $(BChl)_2^{\cdot}$ (Phase II) [15, 4]. As indicated earlier, these two electron transfer reactions together appear to span the membrane ($(BChl)_2$ being near the center of the membrane dielectric), and it has been thought that phases I and II represent the response of carotenoid molecules to electric charge separation, which in this case crosses the membrane dielectric in two distinct steps. The amplitude of the millisecond, antimycin sensitive phase III is similar to the sum of phases I and II, and has been considered [15, 13] to originate from a transmembrane electron transfer and charge separation event within the Q-cytochrome b-c_2 part of the photosynthetic electron flow cycle. However its source of generation is very much open to conjecture.

The ability to employ near-saturating light flashes to elicit single turnovers of the reaction center allows us to count every electron entering and leaving the Q-cytochrome b-c_2 oxidoreductase. The large difference in the rate of the flash induced oxidation of cytochrome c_2 with respect to the subsequent reduction provides a ready way of studying the time-resolved kinetics of the cytochrome c_2 reduction reactions. This technique, combined with redox potentiometry and exploiting the different pH dependencies of the E_m values of the different components (see ref. 16 for a review) has been used in this report to describe the physical chemistry of an important electron donor to cytochrome c_2, and to provide more information on the possible origins of the antimycin-sensitive phase III of the carotenoid bandshift.

MATERIALS AND METHODS

Rhodopseudomonas sphaeroides Ga was grown anaerobically in the light with succinate as sole carbon source and chromatophores were prepared as previously described [4]. Redox potentiometry and the analysis of rapid flash-induced absorbancy changes in a dual wavelength spectrophotometer have also been described earlier [4]. The basic methods and precautions for redox potentiometry are discussed in ref. 17. Particular care was taken to ensure that the concentrations of the mediating redox dyes were sufficient to allow reliable redox equilibration between the components of the chromatophores and the electrodes, but not so high as to significantly interfere with the flash induced kinetics measured in the tens of milliseconds. Kinetic patterns which were obtained with dyes present were always demonstrated in the absence of dyes; an important example is shown in Fig. 1. Addition of small amounts

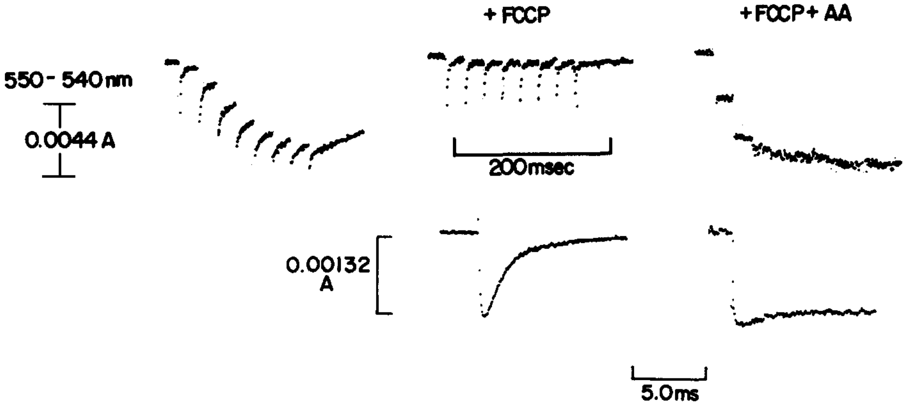

Fig. 1. The response of cytochrome c_2 to a train of single-turnover flashes of light. Chromatophores (23 μM BChl) were suspended in 20 mM morpholinopropane sulfonate (MOPS), 100 mM KCl pH 7.0. No redox dyes were added, but the redox potential was adjusted with sodium dithionite until the ambient redox potential was approximately +50 mV (cf. Fig. 2). The chromatophores were then subjected to a train of 8 single turnover flashes of light with no further additions, and in the presence of 10 μM carbomylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), and 10 μM FCCP plus 2 μM antimycin as indicated. The kinetics are examined in greater detail in the lower part of the figure ([BChl] = 18 μM). The traces represent the average of 32 trains of pulses.

of dithionite, ascorbate or succinate to chromatophores under anaerobic conditions results in the reduction of most of the components in the Q-cytochrome b-c_2 system. Under coupled conditions, flash activation yields the kinetic patterns shown on the left of Fig. 1; cytochrome c_2 is re-reduced in 1–2 ms halftime after the first flash [4, 6] and does not achieve a full state of flash-induced oxidation until after the fourth or fifth flash, when the re-reduction process assumes "energy dependent" values up to seconds in halftime depending on the quality of the coupling status of the chromatophore. Under these reducing conditions, uncoupler has a dramatic effect, allowing 1–2 ms re-reduction of flash oxidized cytochrome c_2 after all flashes; a closer view of the oxidation-reduction kinetics of cytochrome c_2 oxidation-reduction under uncoupled conditions is shown in the lower center of the figure. On the right is shown the effect of antimycin, which extends the re-reduction halftime of cytochrome c_2 into the seconds time range. The inhibitory effect is seen at the first and subsequent turnovers, and the requirement for two separate flash activated turnovers of the reaction center to oxidize the two associated cytochrome c_2 molecules [4, 5] is clearly shown. The lower right trace shows the cytochrome oxidation and inhibited reduction reaction in more detail.

RESULTS

Cytochrome c_2 oxidation-reduction

Fig. 2 shows the response of the cytochrome c_2 complement of the chromatophores to a series of eight near-saturating single-turnover light flashes measured under coupled and uncoupled conditions, as a function of redox potential. On the left of each column are the oxidation-reduction reactions observed in the coupled state,

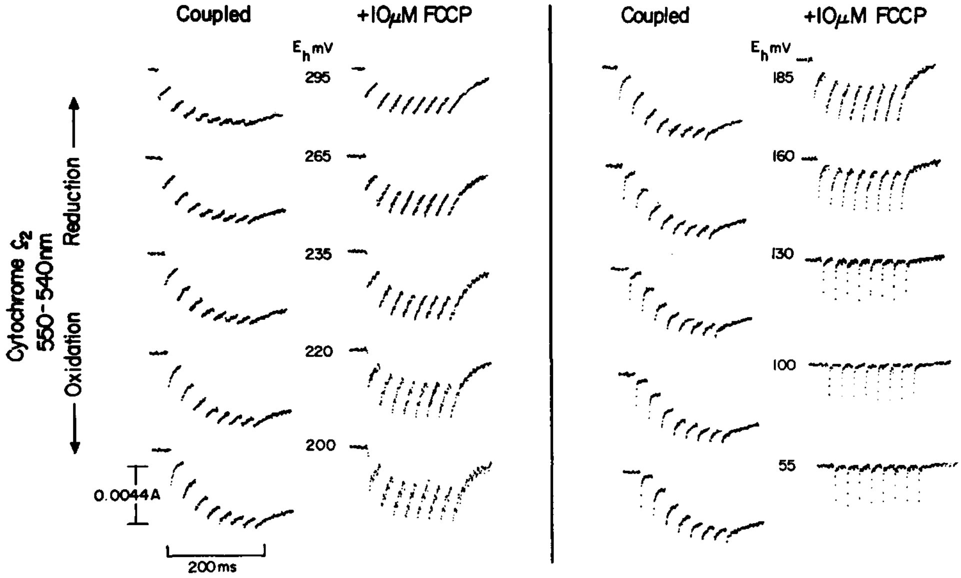

Fig. 2. The response of cytochrome c_2 to a train of single-turnover flashes of light as a function of redox potential. Chromatophores (23 μM BChl) were suspended in 20 mM MOPS, 100 mM KCl pH 6.5 in the presence of 7 μM each of *N*-methylphenazonium methosulfate (PMS), *N*-ethylphenazonium ethosulfate (PES), diaminodurol, pyocyanine and 2-hydroxy-1,4-naphthaquinone as redox mediators. 10 μM FCCP was present where indicated.

where the chromatophores are capable of ATP synthesis. The first trace (top left) was obtained at a redox potential of 295 mV, where some slow (tens or hundreds of milliseconds), but no rapid (i.e., $t_{\frac{1}{2}}$ 1–2 ms) re-reduction of the flash-oxidized cytochrome c_2 is observed after the first flash. After the fourth flash, almost all the re-reduction occurs in the several hundreds of milliseconds to seconds time domain. As the ambient redox potential of the coupled chromatophores is lowered through the 100–200 mV region, 1–2 ms halftime re-reduction of the cytochrome c_2 after the first flash becomes more evident. However, irrespective of the state of reduction of the components of the Q-cytochrome *b*-c_2 oxidoreductase prior to activation, the cytochrome c_2 complement approaches a fully oxidized state after the first four flashes. Examination of the kinetics, for example at an ambient potential of 55 mV, shows that with successive flashes from the first to the fourth, the proportion of the 1–2 ms halftime contribution to cytochrome c_2 reduction decreases, and the second slower reduction process becomes dominant, allowing the cytochrome c_2 to assume a high level of oxidation despite the low ambient potential.

On the right of each column of Fig. 2 are the oxidation-reduction reactions of cytochrome c_2 in chromatophores in the uncoupled state. At high potentials the effect of uncoupler on cytochrome c_2 re-reduction (i.e., uncoupler stimulated electron transfer through the oxido-reductase) is not remarkable; the stimulation is only 3 or 4 fold. However, as the redox potential is lowered from 200 to 100 mV, the difference between the coupled and uncoupled states becomes dramatic. At the lower potential under uncoupled conditions, cytochrome c_2 is re-reduced in 1–2 ms after every flash, and the stimulation due to uncoupler, measured after the fourth flash, is about 1000 fold. Thus the component reduced at equilibrium in the 200 to 100 mV redox potential range not only effects the reduction of cytochrome c_2, but also confers an enhanced sensitivity to uncouplers on the system. In other words, the electron transfer from the donor to cytochrome c_2 seems to be very important for the efficient energy-coupled functioning of the Q-cytochrome b-c_2 oxidoreductase.

In order to assess the electrochemical properties of the millisecond donor to cytochrome c_2, we have measured the extent of oxidation of cytochrome c_2 5 ms after the first flash, as a function of redox potential. This time interval was chosen to permit near completion of the 1–2 ms halftime re-reduction phase, without including much of the slower phases. The data provides the Nernst curve, and hence the E_m and n-

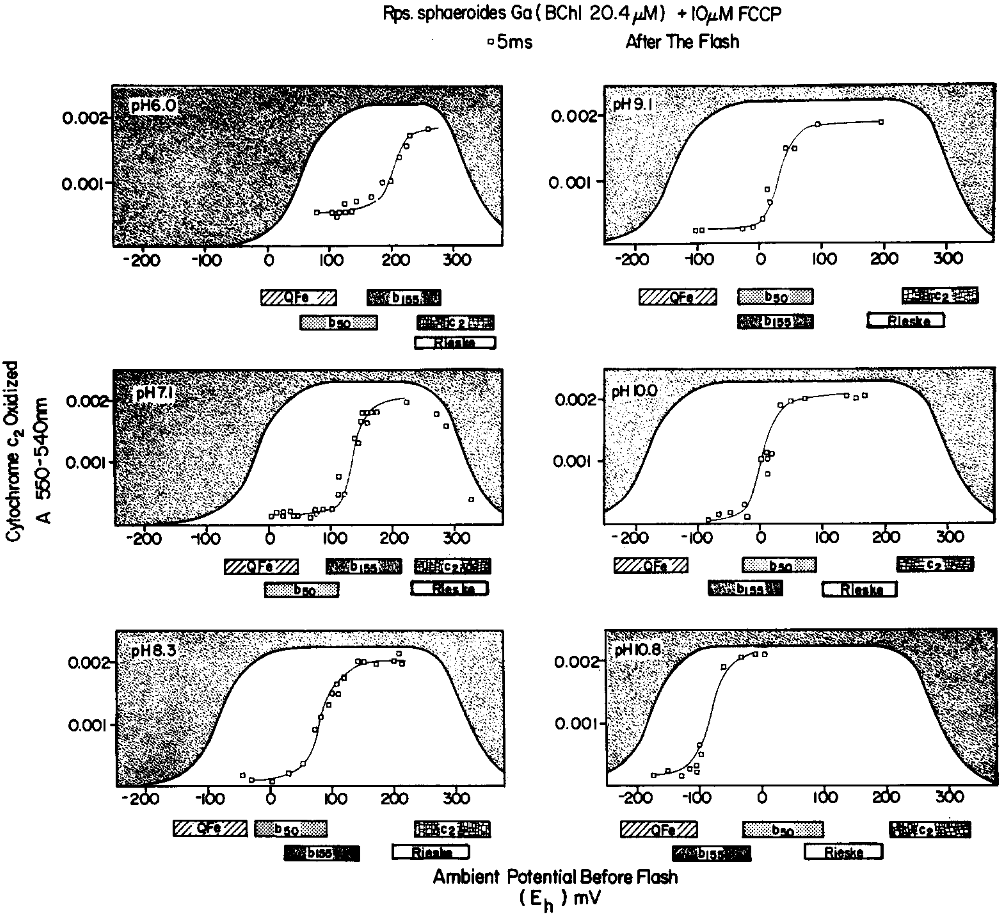

Fig. 3. The redox potential dependency of cytochrome c_2 reduction after a single turnover flash in the uncoupled state. Chromatophores (20.4 μM BChl) were suspended in 100 mM KCl, 20 mM of an appropriate buffer (2(*N*-morpholino)ethanesulfonic acid (MES), MOPS, Tris, glycylglycine or glycine) in the presence of 5 μM each of PMS, PES, diaminodurol, pyocyanine and 2-hydroxy-1,4,-naphthaquinone plus 10 μM FCCP.

values of the millisecond donor in question, and by repeating the titrations at different pH-values, the relationship of the redox couple with protons can also be determined. This information can then be compared to the known properties of the various candidates for the immediate donor to cytochrome c_2.

The first turnover of cytochrome c_2 oxidation-reduction in the uncoupled state

The results are shown in Fig. 3 and the necessary information for this extensive figure will first be discussed for the frame for pH 7.0 (middle left of Fig. 3). The shaded area circumscribes the redox potential dependence of the extent of single turnover flash generated cytochrome c_2 oxidation (i.e., measured in less than 1 ms), and underneath the figure are the known E_m (pH 7.1) values of relevant components. The attenuation of the flash-induced cytochrome c_2 oxidation at high redox potentials decribes the equilibrium course of chemical oxidation of cytochrome c_2 itself [3–6], such that at potentials much greater than the E_m of the cytochrome there is no reduced cytochrome available for flash oxidation. The attenuation of flash-induced cytochrome oxidation at low potentials describes the equilibrium chemical reduction of the reaction center "primary acceptor" QFe [18], which prevents useful flash induced oxidation of $(BChl)_2$, in turn preventing any cytochrome oxidation. In order to simplify the figure, points are not shown on this well-established (eg. ref. 6) line. The points that are shown represent the extent of oxidation measured 5 ms after a single turnover flash. As expected from Fig. 2, at potentials above 250 mV, little immediate re-reduction occurs after the flash, and the majority of the oxidized cytochrome remains oxidized 5 ms after the flash. However, as the potential is lowered from 200 to 100 mV, almost complete re-reduction of the cytochrome occurs within 5 ms. The 5 ms data of

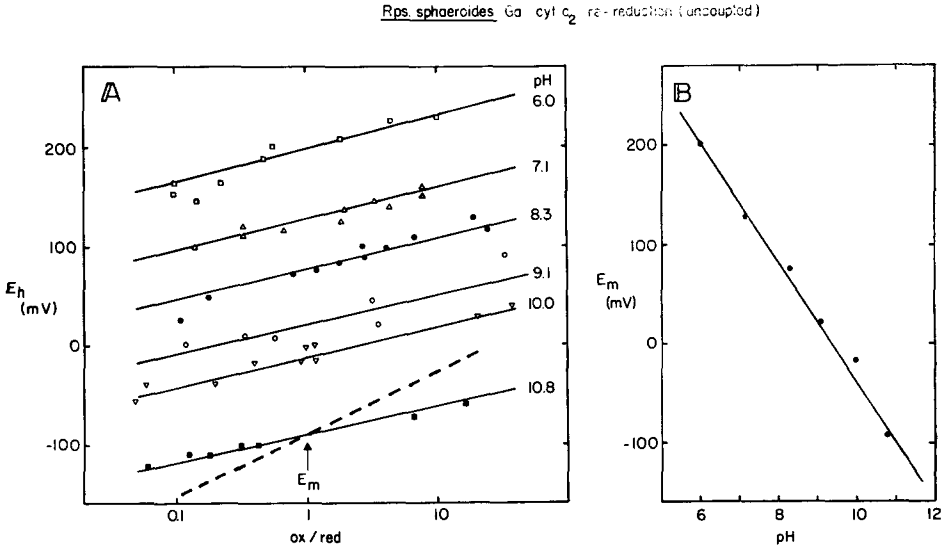

Fig. 4. The redox potential dependency of cytochrome c_2 reduction after a single turnover flash in the uncoupled state. Part A presents the data of Fig. 3 on semi-log plots; the solid lines reflect theoretical lines for an $n = 2$ redox reaction, and the dashed line that for an $n = 1$ reaction. Part B plots the E_m of the donor to cytochrome c_2 as a function of pH.

Fig. 3 thus describe the redox curve of a 1–2 ms electron donor to ferricytochrome c_2, which has an E_m (pH 7.1) of about 140 mV. Examining the other five experiments of Fig. 3, done at pH values from 6.0 to 10.8, it is evident that the 1–2 ms electron donor displays a pH dependent E_m value over the entire range. Fig. 4B presents this more clearly; the E_m of the donor varies with pH by −60 mV/pH unit, indicating that the donor requires one proton per electron for its reduction. The *n*-value of the donor appears to be close to 2.0, and the lines drawn in Fig. 3 are Nernst curves for this *n*-value; for clarity the data are shown on semilog plots in Fig. 4A.

The electron donor to cytochrome c_2 on subsequent turnovers in the uncoupled state

Repeating the experiments described above, but measuring the amount of cytochrome remaining oxidized 5 ms after turnovers subsequent to the first yields the redox potential dependency of cytochrome c_2 re-reduction shown in figure 5. The electron donor to cytochrome c_2 is still apparent as having an E_m of 150 mV (pH 7.0) and an *n*-value of close to 2.0. The data of Fig. 5 were obtained after 32 turnovers, but the result is apparently the same for any turnover (including the first, see Fig. 3). In other words, under uncoupled conditions electron flow through the Q-cytochrome *b*-c_2 oxido-reductase is possible with millisecond halftime kinetics after every turnover, if the 150 mV (pH 7.0) component is reduced before activation.

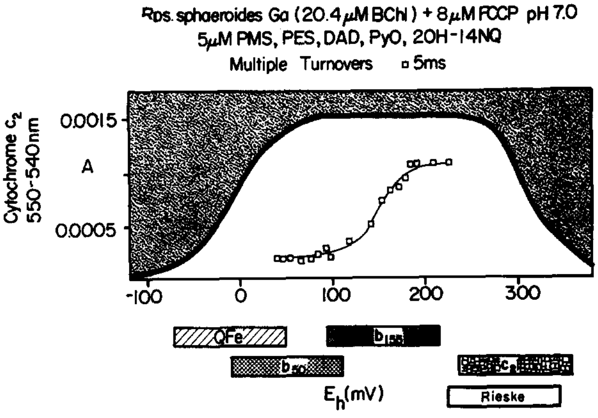

Fig. 5. The redox potential dependence of cytochrome c_2 reduction in the uncoupled state. Chromatophores (20.4 μM BChl) were suspended in 20 mM MOPS, 100 mM KCl, pH 7.0 together with 5 μM each of PMS, PES, diaminodurol, pyocyanine and 2-hydroxy-1,4,-naphthaquinone and 8 μM FCCP. They were then subjected to a train of 32 single-turnover flashes separated by 25 ms, and the responses of cytochrome c_2 to these flashes were averaged (in the same way as in ref. 5). The data represent the amount of cytochrome c_2 remaining oxidized 5 ms after the flashes.

The electron donor to cytochrome c_2 in the coupled state

Fig. 6 shows a redox titration of the donor to cytochrome c_2 following the first turnover of the system in the coupled state: Once again an E_m value (pH 7.0) of about 150 mV with an *n* value of 2 is obtained. The extent of the 8 ms re-reduction of cytochrome c_2 is less in the coupled state, suggesting that there is some energetic feedback to the reduction reaction from the coupling mechanism. This effect becomes more

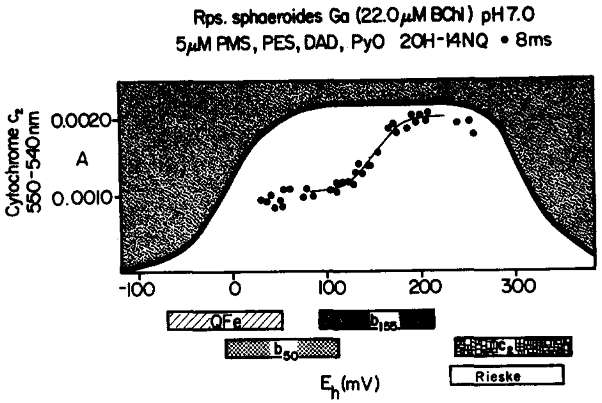

Fig. 6. The redox potential dependence of cytochrome c_2 reduction in the coupled state. Chromatophores (22 μM BChl) were suspended in a medium identical to that for Fig. 5, except that the FCCP was omitted. The data represents the amount of cytochrome c_2 remaining oxidized 8 ms after a single turnover flash.

pronounced with successive flashes in the coupled state (Fig. 2) and the 1–2 ms re-reduction is essentially replaced by a much slower phase after four turnovers. Nevertheless, although the extent of the more rapid re-reduction phase decreases with successive flashes, the thermodynamic properties of the donor seem to be the same as those measured after the first flash.

Thus it may be concluded that the 150 mV (pH 7.0) $n = 2$ component is a major millisecond electron donor to ferricytochrome c_2 on the first and subsequent turnovers in the energy coupled and uncoupled state. Furthermore, it is apparent from Fig. 2 that the component in question, if reduced before activation of the electron (and proton?) translocation through the Q-cytochrome b-c_2 oxidoreductase, exerts a profound effect on the uncoupler sensitivity of the system. A similar effect is seen in the presence of valinomycin (1 μM) and potassium ions, but nigericin (1 μM) has little effect on the re-reduction patterns of cytochrome c_2.

The third phase of the carotenoid bandshift

Similar experiments with coupled chromatophores were repeated with spectrophotometric analysis of the carotenoid bandshift following flash activation. Under all the conditions of E_h and pH examined phase III of the carotenoid bandshift exhibited near constant flash induced formation halftimes in the millisecond time range, and the decay of the shift had halftimes of hundreds of milliseconds to seconds. Fig. 7 shows the extent of the carotenoid bandshift as a function of ambient redox potential before activation. As with the cytochrome c_2 reduction determinations, the measurements were repeated over the pH 6 to 10.5 range. In Fig. 7 the total extent of the shift was measured 5 ms after a single turnover flash and all three phases are clearly resolvable as a function of ambient redox potential at each of the pH values studied. At a redox potential of 400 mV, where only $(BChl)_2$ is reduced before activation, only phase I occurs. At a redox potential of about 200 mV, where cytochrome c_2 is also reduced before activation, both phases I and II are seen: As expected from the pH independencies of the E_m values of cytochrome c_2 and $(BChl)_2$, the redox potential

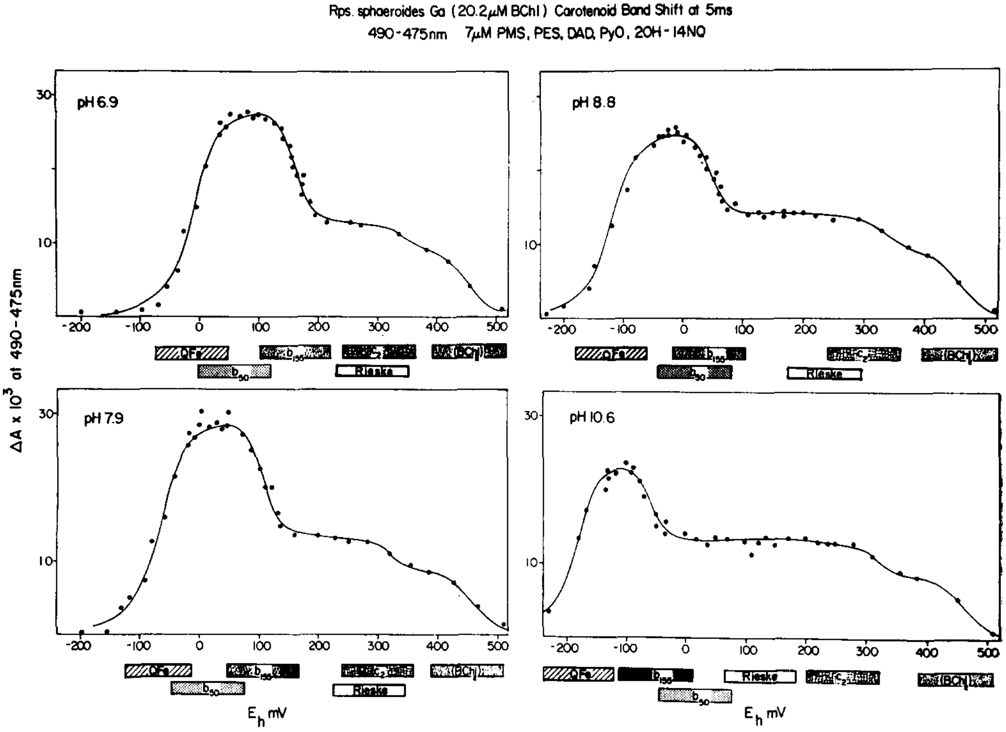
Rps. sphaeroides Ga (20.2μM BChl) Carotenoid Band Shift at 5ms 490-475nm 7μM PMS, PES, DAD, PyO, 2OH-14NQ

Fig. 7. The redox potential dependency of the carotenoid bandshift. Chromatophores (20.2 μM BChl) were suspended in similar buffers to those used in Fig. 2, together with the same redox mediators, but in the absence of FCCP. The extent of the carotenoid bandshift 5 ms after a single-turnover flash of light is plotted as a function of the ambient redox potential.

dependencies of phases I and II are not markedly affected by pH. In contrast, the appearance of phase III as the redox potential is lowered is clearly pH dependent.

The thermodynamic properties of the redox component which allows phase III of the carotenoid bandshift if reduced before flash activation are analyzed in Fig. 8. In the four experiments shown in Fig. 7, together with five other determinations included in figure 8, the flash induced amplitude of phase III titrates as a function of redox potential as if the redox components in question requires two electrons for its equilibrium reduction; that is, it has an n-value close to 2.0 (Fig. 8A). In addition, the E_m of the component varies by -60 mV/pH unit between pH 5.6 and 10.6 (Fig. 8B).

Fig. 9 summarizes the information from both the cytochrome re-reduction and the carotenoid bandshift experiments. On the left, all the experimental data are superimposed on one Nernst semi-log plot, where the data strongly favor the theoretical $n = 2$ rather than the $n = 1$ line. On the right of the figure, the individual E_m values obtained at a variety of values of pH fit a -60 mV/pH unit line over a 10^5 change in hydrogen ion activity across the physiological pH range. These properties, together with the similar 1-2 ms halftime of cytochrome c_2 reduction and carotenoid bandshift phase III formation, and the inhibition by antimycin of both effects, suggest that the same redox component [E_m (pH 7.0) $\approx$ 150 mV, $n = 2.0$] is important for the occurrence of both events.

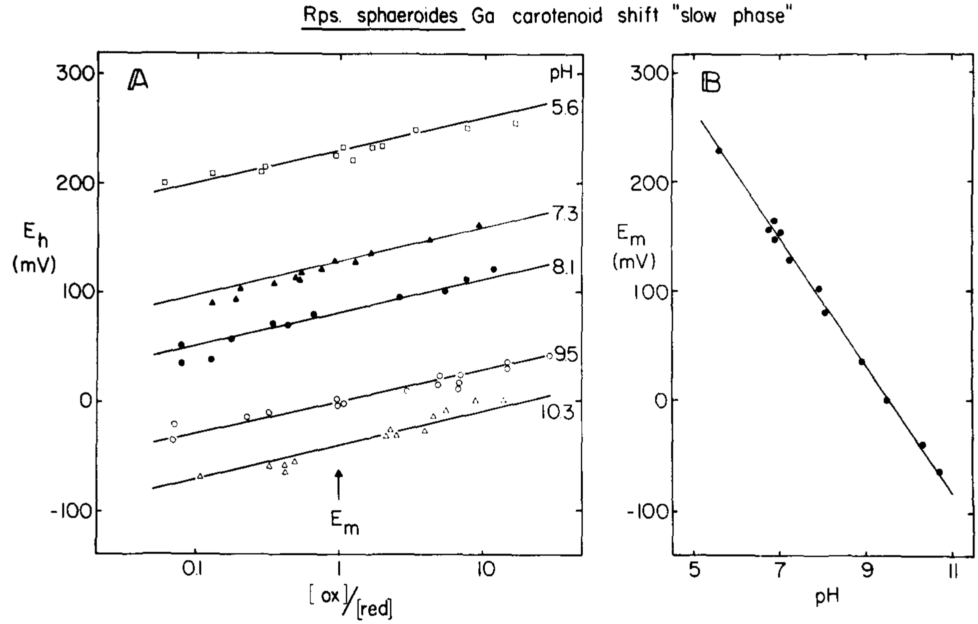

Fig. 8. The redox potential dependence of the carotenoid bandshift phase III or "slow phase". Part A presents experiments similar to those in Fig. 7 performed at different values of pH, and the data are presented on semilog plots where the lines drawn through the points represent the theoretical line for an $n = 2$ redox couple. Part B presents the data of Figs. 7 and 8A as a function of pH.

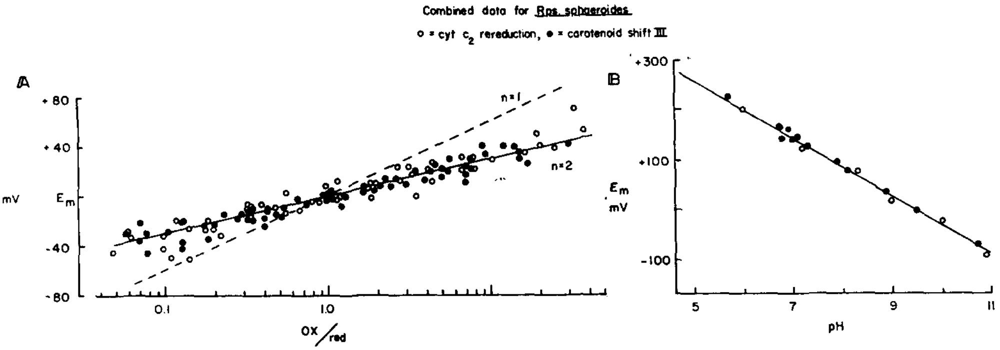

Fig. 9. The redox potential dependency of cytochrome c_2 reduction and the third phase of the carotenoid bandshift. Part A combines the data from Figs. 3, 4, 7 and 8 on a single semilog plot; the solid line represents the theoretical line for an $n = 2$ redox reaction, the dashed line an $n = 1$ reaction. Part B presents the combined data with the E_m plotted as a function of pH.

DISCUSSION

The physical-chemical nature of the electron donor to cytochrome c_2, and criteria for reliability

The component identified with cytochrome c_2 reduction and carotenoid bandshift phase III generation [E_m (pH 7.0) = 155 ± 15 mV] apparently undergoes equilibrium oxidation-reduction from pH 5.8 to 10.9 as follows

$$\mathrm{Red\ H_2} \rightleftharpoons \mathrm{Ox} + 2e^- + 2H^+$$

for which the approximate Nernst expression is

$$E_h = E_m\ (\mathrm{pH}\ x) + 30 \log \frac{[\mathrm{Ox}]}{[\mathrm{Red\ H_2}]} - 60\ \mathrm{pH}\ y - x$$

where E_m (pH x) is the midpoint potential at a specified pH, x, and the pH y is the ambient pH of the redox couple. The parameters of novel interest are the n value and the ability to carry protons. Although we can be fairly certain of the measured E_m value of the couple as almost always falling within 15 mV of the measured mean value (and hence can be certain of the pH dependency of the E_m) we are aware of the pitfalls associated with n-value determinations in biological systems. However, practically all the titrations of either cytochrome c_2 re-reduction or the third phase of the carotenoid bandshift fitted the simple $n = 2$ line rather well, regardless of whether the data were measured 5 or 25 ms after the flash. This reproducibility is also seen in previously published titrations of the cytochrome c_2 reduction [6] and the carotenoid bandshift [15]. Similar results are obtained when the redox mediating dyes are varied in concentration from 4 to 25 μM, and the titrations are readily reversible.

The possible identity of the electron donor to cytochrome c_2, and the other redox components of the Q-cytochrome b-c_2 oxidoreductase

In the Introduction we briefly outlined the several components present in chromatophores which could possibly act as electron donors to ferricytochrome c_2. The data presented in this paper allow us to reconsider their ability to fulfill such a role.

(*a*) *The Rieske g 1.90 iron-sulfur protein.* No evidence was observed in the cytochrome c_2 re-reduction titrations that this component is able to interact with flash-oxidized cytochrome c_2. Its E_m at pH 7.0 is 280 mV [8, 19], which is very similar to that of cytochrome c_2 [4–6]. However the pK on the oxidized form of the Rieske iron-sulfur protein at pH 8 [19] makes its E_m pH-dependent above this pH, so that at pH 10.8 the E_m is 110 mV. This is some 170 mV lower than that of cytochrome c_2, and had the Rieske center been capable of rapid electron donation to flash-oxidized cytochrome c_2, it should have been clearly evident in Fig. 3 at the higher pH values.

(*b*) *The g 1.94 E_m (pH 7.0) 40 mV iron sulfur protein.* The E_m/pH relationship of this redox component has not yet been investigated, but recent experiments [20] indicate that this component is part of the succinic dehydrogenase complex attached to the outside of the chromatophore membrane. As such it is readily removed by fairly mild procedures [20], and cytochrome c_2 re-reduction and the third phase of the carotenoid bandshift are unimpeded by its absence. As such, the E_m (pH 7.0) 40 mV g 1.94 iron sulfur protein does not seem to be involved in the Q-cytochrome b-c_2 oxidoreductase.

(*c*) *Cytochrome b_{50} (b-560).* At neutral and lower pH values, the E_m of cytochrome b_{50} is below that of cytochrome b_{155}, so it cannot be examined uniquely as a potential direct electron donor to cytochrome c_2. However, its pK on the reduced form at pH 7.4 [7] renders its E_m pH-independent at pH values higher than pH 7.4, while that of cytochrome b_{155} apparently remains pH-dependent [7]. Thus at alkaline pH the E_m of cytochrome b_{50} becomes more positive than that of cytochrome b_{155}, so cytochrome b_{50} can then be examined as a direct electron donor to cytochrome c_2 (eg. at E_h 0 mV at pH 10.8). Under these conditions (Fig. 3) there was no evidence that cytochrome b_{50} could directly donate an electron to ferricytochrome c_2.

(*d*) *Cytochrome b_{155}* (*b*-558). The 1–2 ms electron donor to cytochrome c_2 has a very similar E_m value and pH-dependency (Fig. 9) to that of cytochrome b_{155} [7]. The only difference between the kinetically identified electron donor and cytochrome b_{155} is the *n*-value; evidence to date from standard redox titrations of the *b*-cytochromes, followed by mathmatical resolution of the three electrochemical species, indicates an *n* value of unity for cytochrome b_{155} [6, 7].

Current opinions do not place cytochrome b_{155} in the cyclic electron transport chain (see ref. 16); instead it has been proposed to act with cytochrome c_2 or $(BChl)_2$ as perhaps a non-cyclic, substrate linked, energy-uncoupled supplier of reducing equivalents to the cycle [7], although there is little firm evidence to support this suggestion. What has been interpreted as flash-induced cytochrome b_{155} oxidation [6] apparently occurs only after the first turnover, even under uncoupled conditions; in contrast the effects of the prior reduction of the 1–2 ms electron donor to cytochrome c_2 are felt on the first and every subsequent turnover. The functional involvement of cytochrome b_{155} is also open to question on the grounds that its content in chromatophores is widely variable, from undetectable to 35 % of the total *b*-cytochrome complement. Nevertheless, analagous cytochromes are found in *Rhodospirillum rubrum* [6] and *Rps. capsulata* (Dutton, P. L., van den Berg, W. H. and Prince, R. C., unpublished observations), while a similar cytochrome (E_m (pH 7.2) 120 mV) is also seen in pigeon heart submitochondrial particles (the analagous particles to chromatophores). However, this cytochrome is not readily resolved in the parent mitochondria, although it has been reported to occur in beef heart mitochondria [21–23]. In view of these discrepancies, we feel that there is little evidence to equate cytochrome b_{155} with the electron donor to cytochrome c_2, but further work on the *b* cytochromes may clarify the situation.

(*e*) *Ubiquinone-10*. No direct measurements have been made on Q in chromatophores but some information has been inferred from single turnover experiments. The E_m of the primary acceptor Q, which is in close association with an iron atom, is known for both the unprotonated $QFe/Q^{\cdot-}Fe$ (E_m −180 mV, ref. 18) and the protonated $QFe/Q^{\cdot}HFe$ (E_m (pH 7.0) −20 mV, see ref. 18) couples. There is a p*K* on the reduced form [$Q^{\cdot-}Fe/Q^{\cdot}HFe$) at pH 10, but throughout the pH 6–11 range the couple always seems to involve only the fully oxidized and the semi-reduced (semi-quinone) species, having an *n* value close to unity [18].

The majority of the Q complement (perhaps 15 of the approximately 25 ubiquinones per reaction center; Takamiya, K. and Dutton, P. L. unpublished observations) apparently acts as a "pool" to receive electrons from the reaction center, and these bind protons from the external aqueous phase (ref. 2, and Takamiya, K. and Dutton, P. L., unpublished observations). Despite intensive studies [2], the thermodynamic properties of these quinones remain incompletely described, and the E_m values (for the $Q/Q^{\cdot}H$ and the $Q^{\cdot}H/QH_2$ couples) and *n* value(s) are not known. If these ubiquinones did operate as an $n = 2$ Q/QH_2 couple, they would be an attractive candidate as the electron donor to cytochrome c_2, but whether it is this complement (as it would in Mitchell's "Q-cycle" models, see refs. 24–26) or a second distinct complement which might fulfill this role is open to question. Of course a flavin (also usually $n = 2$) is an equally good candidate as the electron donor to cytochrome c_2. In the absence of any firm identification, we shall refer to the component responsible for cytochrome c_2 re-reduction and the third phase of the carotenoid bandshift, as Z.

There is no evidence that Z is present in the membrane as a pool, and indeed the data that we do have are against such an idea. The rates of cytochrome c_2 re-reduction and carotenoid bandshift phase III formation remain fairly constant as the potential is lowered through the 200 to 100 mV range; only the extents of the changes increases. If there was a pool of potential reductants for cytochrome c_2, we might expect the rates of the reactions to become faster, with a first order dependency on the number of reduced donors. However, this expectancy is contingent on the absence of any extra rate limiting steps in the reactions being measured.

Another aspect of the possibility of a pool of kinetically and thermodynamically equivalent electron donors to cytochrome c_2 is that the measured E_m in the experiments reported here would be higher than the true E_m of the pool. This is because the measurements made here involve only a single turnover of the system, and thus require only one member of the "pool" to be reduced per cytochrome c_2 to effect the reduction of the flash oxidized ferricytochrome. This would produce a deviation from the normal symmetrical Nernst curve, and the course of re-reduction of the single turnover generated ferricytochrome c_2 would follow a curve of $1-(1-x)^N$, where x is the fraction of the total complement reduced at a specified E_h, and N is the number of identical molecules in the pool. For example, if there were ten identical members of the pool ($N = 10$) the true E_m (pH 7.0) would be 34 mV lower than the measured value of 155 mV and the course of cytochrome c_2 re-reduction as the potential is lowered would start off somewhat less steeply than an $n = 2$ $N = 1$ curve, and then become much steeper as it approached the measured "midpoint". The experimental scatter of our data does not allow us to conclusively eliminate this possibility, but at the same time the data do not support it.

The reaction of a two electron, two proton redox carrier with a one electron, no proton requiring cytochrome c_2

The reaction is

$$ZH_2 + \text{ferricytochrome } c_2 = Z^{\bullet}H + H^+ + \text{ferrocytochrome } c_2$$

assuming that the pK of $Z^{\bullet}H_2^+/Z^{\bullet}H$ is low while that of $Z^{\bullet}H/Z^{\bullet -}$ is high. The E_m of the $Z^{\bullet}H/Z$ couple could be significantly lower than the average $n = 2$ couple (see reference 27 for a full treatment of this kind of reaction). In other words the oxidation of ZH_2 by cytochrome c_2 could produce a single electron reductant; this type of reaction has been the basis for one of Mitchell's recently proposed "Q-cycles" [24–26]. It is conceivable that $Z^{\bullet}H$ serves to reduce, perhaps via intermediary electron carriers near the innerside of the membrane, a component nearer the outerside of the chromatophore membrane. If this transmembrane reductive step involved only an electron, and was thus electrogenic, it could be the source of the third phase of the carotenoid bandshift. The cycle could then be completed by the reaction of the outer Q complement with the inner Z/ZH_2 couple, and this is shown schematically in Fig. 10. Presented in this way, it is similar to one of the Q-cycles of Mitchell [24–26], although we propose two distinct parts (Q and Z) to the proton translocating system, with limitations on the ability of the outer Q to react directly with cytochrome c_2. Although other schemes can be

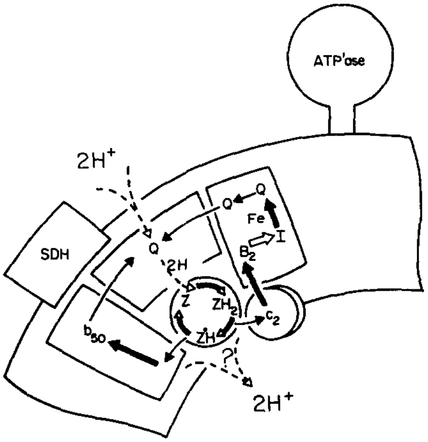

Fig. 10. A model for the Q cytochrome b-c_2 oxido-reductase of *Rps. sphaeroides*. This figure, which is discussed in the text, shows one model which can be drawn from the data presented in this paper. The electron donor (Z) to cytochrome c_2 is shown here as the Z/ZH_2 couple, and the oxidation of this by ferricytochrome c_2 is shown as producing Z˙H. This in turn might reduce an unknown component near the inside of the chromatophore membrane (perhaps cytochrome b_{-90}, as discussed in ref. 16) which could then reduce cytochrome b_{50} near the outerside of the membrane, and in so doing generate the third phase of the carotenoid bandshift. In this figure the thick arrows represent electron transport steps which generate the carotenoid bandshift and B_2 represents $(BChl)_2$. Further details on the transmembrane reactions of the reaction center can be found in ref. 1, on the location of cytochrome c_2 and the ATPase in ref. 3 and on the location of the succinate dehydrogenase complex in ref. 20.

concocted to explain the experimental data (e.g. see refs. 13 and 16, where several are discussed) the model of Fig. 10 is currently one of the simplest. In the absence of reduced Z, there would be no reductant for ferricytochrome c_2, and no reductant for the electron transfer step which generates phase III of the carotenoid bandshift. This suggests a site of action for the inhibitor antimycin, for if this prevented the reduction of ferricytochrome c_2 by ZH_2, it would also prevent the formation of phase III of the carotenoid bandshift. The "W" shaped scheme also explains several other phenomena which are difficult to reconcile with linear schemes, such as that suggested in ref. 7. For example the carotenoid bandshift is stable for seconds at all values of pH, despite the fact that proton uptake by the secondary quinone pool does not occur at alkaline pH [2]: Linear schemes would predict that if no protons were picked up by the secondary acceptors, the completion of the cycle would result in the collapse of the membrane potential generated by the light reaction as the electron (without a proton) recrossed the membrane to reduce ferricytochrome c_2. The model of figure 10 readily accommodates the stability of the carotenoid bandshift by assuming that the reduction of Z to ZH_2 is prevented by the absence of proton binding to the outer membrane.

Several questions of course remain, in particular we are uncertain of the fate of the protons on ZH_2, and indeed about how they get to ZH_2 in the first place. It is noteworthy that for the first few turnovers of the system, the effects of valinomycin on cytochrome c_2 reduction and the carotenoid bandshift are indistinguishable from those of uncoupler, while nigericin is apparently without effect. This suggests that at least for the first few turnovers, the major energetic feedback on electron transport is membrane potential, and not a pH gradient. Does this imply that the protons are not released into the inner aqueous phase, but rather into some location where they are inaccessible to nigericin? Another currently unanswered question is why a membrane potential should affect the apparently electroneutral reduction of Z to ZH_2, for it is this reduction which seems to be the rate-limiting step under energy-coupled conditions. Experiments aimed at answering these questions are currently underway.

Relationship with the mitochondrial Q-cytochrome b-c_1 (c) oxidoreductase

The *Rps. sphaeroides* Q-cytochrome b-c_2 oxido-reductase has many similarities to the mitochondrial system, both in the redox components involved (e.g. see 17, although the *Rps. sphaeroides* system lacks an analog of the mitochondrial cytochrome c_1) and in the membrane location of the components. Several years ago, Baum et al. [28] suggested the existence of an electron carrier, which they termed X, operating between mitochondrial cytochromes b and c_1, and Leung and Hinkle [29] suggested a carrier Z in the same location; more recently Mitchell has discussed the possible involvement of ubiquinone in some detail [24–26]. In many respects these would be analogous to the component Z described here.

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REFERENCES

1 Dutton, P. L., Prince, R. C., Tiede, D. M., Petty, K. M., Kaufmann, K. J., Netzel, T. L. and Rentzepis, P. M. (1977) Brookhaven Symp. Biol. 28, 213–237
2 Petty, K. M. and Dutton, P. L. (1976) Arch. Biochem. Biophys. 172, 335–345
3 Prince, R. C., Baccarini-Melandri, A., Hauska, G. A., Melandri, B. A. and Crofts, A. R. (1975) Biochim. Biophys. Acta 387, 212–227
4 Dutton, P. L., Petty, K. M., Bonner, H. S. and Morse, S. D. (1975) Biochim. Biophys. Acta 387, 536–556
5 Prince, R. C. and Dutton, P. L. (1977) Biochim. Biophys. Acta 459, 573–577
6 Dutton, P. L. and Jackson, J. B. (1972) Eur. J. Biochem. 30, 495–510
7 Petty, K. M. and Dutton, P. L. (1976) Arch. Biochem. Biophys. 172, 346–353
8 Prince, R. C., Lindsay, J. G. and Dutton, P. L. (1975) FEBS Letts. 51, 108–111
9 Takamiya, K-I., Nishimura, M. and Takamiya, A. (1967) Plant Cell Physiol. 8, 79–86
10 Peters, G. A. and Cellarius, R. A. (1972) Biochim. Biophys. Acta 256, 544–547
11 Urban, P. F. and Klingenberg, M. (1969) Eur. J. Biochem. 9, 519–529
12 Cogdell, R. J., Jackson, J. B. and Crofts, A. R. (1972) Bioenergetics 4, 413–429
13 Crofts, A. R., Crowther, D. and Tierney, G. V. (1975) in Electron Transport Chains and Oxidative Phosphorylation (Quagliariello, E. et al., eds.), pp. 233–241, North-Holland
14 Prince, R. C. and Dutton, P. L. (1975) Biochim. Biophys. Acta 387, 609–613
15 Jackson, J. B. and Dutton, P. L. (1973) Biochim. Biophys. Acta 325, 102–113
16 Dutton, P. L. and Prince, R. C. (1977) in The Photosynthetic Bacteria (Clayton, R. K. and Sistrom, W. R., eds.), Plenum Press, New York
17 Dutton, P. L. and Wilson, D. F. (1974) Biochim. Biophys. Acta 346, 165–212
18 Prince, R. C. and Dutton P. L. (1976) Arch. Biochem. Biophys. 172, 329–334
19 Prince, R. C. and Dutton, P. L. (1976) FEBS Letts. 65, 117–119
20 Ingledew, W. J. and Prince, R. C. (1977) Arch. Biochem. Biophys. 178, 303–307

21 Dutton, P. L., Wilson, D. F. and Lee, C. P. (1970) Biochemistry 9, 5077–5082

22 Lindsay, J. G., Dutton, P. L. and Wilson, D. F. (1972) Biochemistry 11, 1937–1943

23 Davis, K. A., Hatefi, Y., Poff, K. L. and Butler, W. L. (1973) Biochim. Biophys. Acta 325, 341–356

24 Mitchell, P. (1975) FEBS Letts. 56, 1–6

25 Mitchell, P. (1975) FEBS Letts. 59, 137–139

26 Mitchell, P. (1976) J. Theor. Biol. 62, 327–367

27 Clark, W. M. (1960) Oxidation-Reduction Potentials of Organic Systems, Williams and Wilkins, Baltimore

28 Baum, H., Rieske, J. S., Silman, H. I. and Lipton, S. H. (1967) Proc. Natl. Acad. Sci. U.S.A. 57, 798–805

29 Leung, K. H. and Hinkle, P. C. (1975) J. Biol. Chem. 250, 8467–8471