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A REEVALUATION OF THE EVENTS LEADING TO THE ELECTROGENIC REACTION AND PROTON TRANSLOCATION IN THE UBIQUINOL-CYTOCHROME *c* OXIDOREDUCTASE OF *RHODOPSEUDOMONAS SPHAEROIDES*

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Chromatophore membranes from *Rhodopseudomonas sphaeroides* activated by light display a carotenoid band shift (phase III) that occurs in response to the electrogenic event (charge separation) in the ubiquinol-cytochrome *c* oxidoreductase. The rate of formation of this electrogenic event has previously been shown to be strongly dependent on the initial redox state of a bound ubiquinone species (designated Q_Z) associated with the oxidoreductase. When Q_Z is reduced (quinol form; Q_ZH_2) the electrogenic event takes place in less than 5 ms. When Q_Z is oxidized (quinone form; Q_Z) it is much slower; under these conditions the fact that it occurs has been ignored. In this report, we address this issue and describe events that lead to the generation of carotenoid band shift phase III when the total population of Q_Z of the chromatophore is oxidized before flash activation. The following characteristics are apparent: (1) When oxidized Q_Z is present before activation, the half-time of formation of carotenoid band-shift phase III is 10–20-times slower than when Q_ZH_2 is present before activation. (2) When oxidized Q_Z is present, the measured full extent of phase III generated by a single-turnover flash is diminished by about one-half of that observed when Q_ZH_2 is present before activation. (3) The rate of formation of the carotenoid band shift phase III when Q_Z is initially oxidized corresponds closely to the rate of completion of the flash-activated electron-transfer cycle. This can be seen under two different conditions: (a) as the partial reduction of cytochrome $c_1 + c_2$ (at redox potentials of 200–300 mV) or (b) as the partial reduction of flash-oxidized bacteriochlorophyll dimer, $(BChl)_2^+$ (at redox potentials above 300 mV). (4) At the higher redox potentials (above 300 mV), antimycin-sensitive proton binding shares a common, rate-limiting step with the carotenoid band shift phase III and $(BChl)_2^+$ reduction. (5) However, proton binding at redox potentials above 300 mV is not observed at all unless valinomycin (K^+) is present. Thus, proton binding occurs only when the carotenoid band shift is collapsed in milliseconds, whereas, conversely, the carotenoid band shift is stably generated when proton binding is not observed. These and other observations are the basis of a reevaluation of our current views on the coupling of electron transfer and proton translocation in photosynthetic bacteria.

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Abbreviations: BChl, bacteriochlorophyll; Mops, 4-morpholinepropanesulfonic acid; DAD, 2,3,5,6-tetramethylphenylenediamine.

Introduction

It is currently accepted that the principal function of the ubiquinol-cytochrome *c* oxidoreductase (cytochrome *b*-c_1 complex) of the photosynthetic membrane of *Rhodopseudomonas sphaeroides* is the generation of an electrochemical proton gradient

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across the membrane. A single ubiquinone molecule (Q_Z) associated with ubiquinol-cytochrome *c* oxidoreductase plays an essential role in this process. The role has been largely defined by the pronounced influence of the redox state of Q_Z at the time of activation upon the rate of electron transfer through the ubiquinol-cytochrome *c* oxidoreductase [1–4].

The ubiquinol-cytochrome *c* oxidoreductase is a major part of the light-driven, cyclic electron-transfer system which is composed of the photochemical reaction center, cytochrome c_2 and the ubiquinol-cytochrome *c* oxidoreductase. It is now well known that following a single-turnover flash this cycle displays three readily distinguishable phases of carotenoid band shifts which are considered to respond to alterations in electric field caused by charge separations directed accross the membrane. The two faster phases (phases I and II, $t_{1/2} < 1$ μs and approx. 0.1 ms, respectively) have been correlated with electron transfers directed across the membrane associated with the reaction center [5,6]. The third phase (phase III) is much slower than phases I and II and is associated with the ubiquinol-cytochrome *c* oxidoreductase. Under optimal conditions (Q_Z poised in its fully reduced, quinol form (designated Q_ZH_2) before activation), the kinetics of carotenoid band shift phase III formation ($t_{1/2}$ 1–2 ms) correspond to those of ferricytochrome c_2 reduction [7] and ferrocytochrome *b*-560 oxidation [1,8,9]; furthermore, like these reactions the carotenoid band shift is inhibited by antimycin. Under optimal conditions the amplitude of phase III is similar to the sum of phases I and II [5,7].

Under conditions that would be considered not optimal (Q_Z in its fully oxidized, quinone form (designated 'oxidized Q_Z') before activation), the flash-activated formation of band-shift phase III is very much slower [5,8,10]. Because of this, studies of the formation of phase III when oxidized Q_Z is present have not been pursued and for the most part the reactions that lead to carotenoid band shift phase III under these conditions have not been considered.

In this report, we described electron-transfer reactions associated with the ubiquinol-cytochrome *c* oxidoreductase which can lead to the generation of significant values of membrane potential and catalyze the translocation of protons under conditions where Q_Z is oxidized prior to flash activation. The work addresses questions on the role of Q_Z in the electrogenic and proton-translocating reactions, and on how the initial redox state of Q_Z may alter this role. A further apparent anomaly is revealed in measurements on the relationship of the coupling of electron transfer to proton translocation; this and other inconsistencies in proton measurements are summarized and discussed.

Materials and Methods

Chromatophores were prepared from photosynthetically grown *Rps. sphaeroides* Ga as described previously [6]. Redox potentiometry and the analysis of rapid flash-induced absorbance changes in a dual-wavelength spectrophotometer have also been described earlier [2,6,7]. The use of *N*-methylphenazonium methosulfate and *N*-ethylphenazonium ethosulfate as redox mediators was avoided in this study because the presence of either accelerates the decay of the carotenoid absorbance change and the rereduction of cytochrome $c_1 + c_2^*$ in the hundreds of milliseconds time scale of our investigations. Reaction center concentrations were calculated from the concentration of photooxidizable $(BChl)_2$ ($\Delta\epsilon_{red-ox} = 29.8\ mM^{-1} \cdot cm^{-1}$ at 605 − 540 nm [6]). Proton-binding measurements with a pH indicator, chlorophenol red, were calibrated from the absorbance change due to the addition to the chromatophore suspension of a small amount of standardized HCl.

Three principal redox states of the system were

* It has recently been shown that two types of cytochrome *c* are functional in photosynthetic electron transfer in *Rps. sphaeroides* [11,12]. One is the water-soluble, membrane-dissociable cytochrome c_2 which reduces $(BChl)_2^+$ directly and the other is a membrane-bound component analogous to mitochondrial cytochrome c_1. Although the measurement with the difference absorbance, 550 − 540 nm, contains contributions from both *c*-type cytochromes, the two cytochromes have similar midpoint potentials and are in rapid equilibrium (less than 1 ms, see Ref. 12). Therefore, the 550 − 540 nm absorption change is designated as the cytochrome $c_1 + c_2$ change in this paper.

studied: One, obtained by establishing a redox potential (E_h) of 100 mV (pH 7.0), involved Q_Z in its reduced, Q_ZH_2 form; these are conditions that promote optimal, rapid electron transfer through the ubiquinol-cytochrome *c* oxidoreductase and served as a comparison for the studies done when Q_Z was oxidized. The two redox states studied which included Q_Z in its oxidized form were as follows. (1) At an E_h of 200–225 mV at which all the cytochromes *b* and Q_Z are oxidized while the Rieske FeS Center, cytochrome c_1 and c_2 and $(BChl)_2$ are reduced: Under these conditions following flash activation, $(BChl)_2$ undergoes oxidation and submillisecond reduction by the immediate electron donors, cytochrome $c_1 + c_2$. After redox equilibration with the Rieske FeS center, the reduction of the remaining ferricytochrome $c_1 + c_2$ is slow due to the lack of other reduced electron donors in the ubiquinol-cytochrome *c* oxidoreductase. (2) At an E_h of 350 mV at which all components of the system (cytochrome $c_1 + c_2$ approx. 90%) are oxidized except $(BChl)_2$: Under these conditions following flash activation, $(BChl)_2$ undergoes oxidation and slower reduction because of the absence of any immediate electron donors.

Results

The kinetics and amplitudes of the carotenoid band shifts with the ubiquinol-cytochrome c oxidoreductase poised at different states of oxidation

Fig. 1 compares the amplitudes and kinetics of the flash-induced carotenoid band shift with the oxidation-reduction kinetics of cytochrome $c_1 + c_2$ (E_h 250 mV) and $(BChl)_2$ (E_h 350 mV). The addition of antimycin, an inhibitor of electron transfer in the ubiquinol-cytochrome *c* oxidoreductase, reveals that a significant fraction of the reducing equivalents that reduce the flash-generated ferricytochrome $c_1 + c_2$ at 250 mV or $(BChl)_2^+$ at 350 mV come through the ubiquinol-cytochrome *c* oxidoreductase. Kinetic contributions from other sources, such as endogenous donors or redox mediators, are minor and, on the tens of milliseconds time scale of the experiment, are readily subtracted. The kinetics of the antimycin-sensitive reduction of ferricytochrome $c_1 + c_2$ or $(BChl)_2^+$ match well with the slow phase of the carotenoid band shift (similarly antimycin sensitive) observed under the same conditions. Thus, we recognize that a significant extent of slow, antimycin-sensitive carotenoid band shift is generated under conditions when the entire Q_Z population of the chromatophore is oxidized. The formation kinetics, being slow, are subjected to the competing and opposing effects of carotenoid band shift decay due to the electrophoretic movement of ions through the membrane. This cannot be ignored on the time scale of hundreds of milliseconds; however, such decay is still relatively slow ($t_{1/2}$ approx. 2 s) as judged from the time course of decay of the rapidly formed phases I and II seen in the presence of antimycin. In the 100–200 ms time required for maximum formation of the slow carotenoid band shift, it was usual that less than 15% of the band shift would decay due to electrophoretic effects.

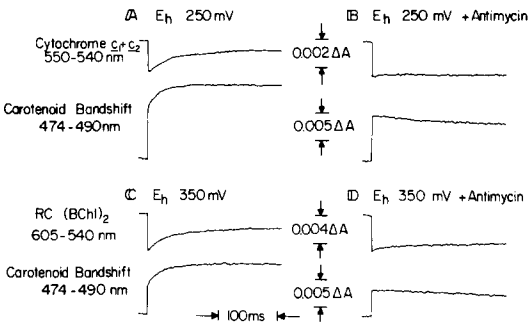

Fig. 1. The kinetics of carotenoid band shift formation, and photooxidized cytochrome $c_1 + c_2$ or $(BChl)_2$ rereduction. Chromatophores ([BChl] 20 μM) were suspended in 20 mM Mops, 100 mM KCl, 0.1 mM FeEDTA (3.3 mM), 5 μM DAD, and 5 μM pyocyanine. The pH was 7.5. Antimycin added was 2 μM. RC, reaction center.

In order to compare the maximum amplitude of the slow carotenoid band shift generated at high E_h values with that seen under optimal conditions when Q_ZH_2 is present, the redox potential dependence of the time-resolved carotenoid band shift was measured; this is shown in Fig. 2. The titration depicted in Fig. 2 was done at pH 9.4 for purpose for demonstration to obtain a clear E_h separation between phases II and III which follow the respective Nernst oxidation-reduction curves of cytochrome $c_1 + c_2$ (E_m nearly pH independent; $E_{m,9.4} \approx 300$ mV) and Q_Z ($E_m - 60$ mV/pH unit; $E_{m,9.4}$ 10 mV) [2]. With Q_ZH_2 present, the flash-induced carotenoid band shift phase III is complete

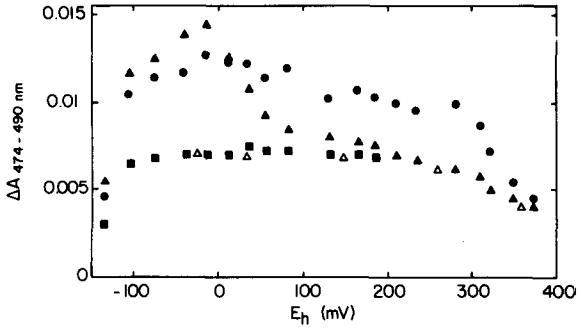

Fig. 2. The time-resolved flash-induced carotenoid band shift. The pH was 9.4 and the buffer used was 20 mM glycine. Other conditions were as in Fig. 1. The data plotted are the extents of the changes at 0.2 ms (■), 5 ms (▲), 100 ms (●) after flash activation. (△) Change with 2 μM antimycin present, 5 ms after the flash.

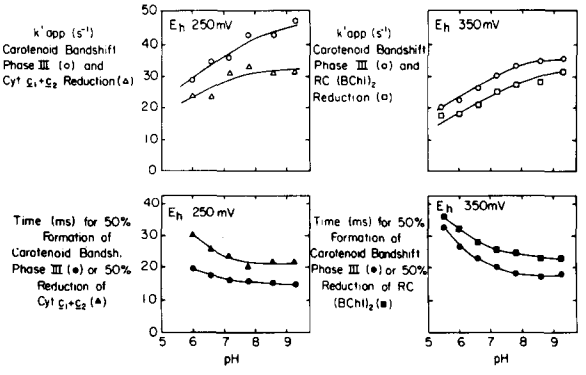

Fig. 3. The effect of pH on carotenoid band shift phase III and electron transfer through the ubiquinol-cytochrome *c* oxidoreductase. Chromatophores (20 μM BChl) were suspended in 100 mM KCl, 50 μM $K_4Fe(CN)_6$, and 10 μM DAD. The pH was adjusted with KOH or HCl. RC, reaction center.

in less than 5 ms. The amplitude measured under these conditions (▲) comprises phases I–III. Addition of antimycin (△) eliminates phase III leaving phases I and II. As expected, this measure of phases I and II merges with that obtained in 0.2 ms (■), which time resolves phases I and II from that of phase III without the aid of antimycin. Phase II can be seen to disappear as the E_h is raised through the 300 mV range (cytochrome $c_1 + c_2$ becomes oxidized); the disappearance of phase I follows the Nernst oxidation-reduction curve (not included in the figure) of $(BChl)_2$ (E_m 450 mV). In agreement with earlier work with Q_ZH_2 present, the flash-induced carotenoid band shift phase III, complete in under 5 ms, is of about the same amplitude as that of the sum of phases I and II measured at 0.2 ms or at longer times in the presence of antimycin.

In comparison, when oxidized Q_Z is present, nearly 100 ms are necessary for phase III to attain its full extent as expected from Fig. 1. The rates obtained for carotenoid band shift phase III formation when Q_Z is oxidized (E_h 250 mV) are 10- and 20-fold slower at pH 6 and 9, respectively, than those measured under similar conditions with Q_ZH_2 present before activation [7]. The extent of phase III when oxidized Q_Z is present is about one-half of the phase III observed under optimal redox conditions when Q_ZH_2 is present.

We have examined in more detail the kinetic relationships between the carotenoid band shift phase III formation and ferricytochrome $c_1 + c_2$ or $(BChl)_2^+$ reduction. Fig. 3 shows the effect of pH on these reactions. Although the apparent rate of phase III formation is always somewhat faster than the rate of completion of the electron-transfer cycle (as assayed by ferricytochrome $c_1 + c_2$ or $(BChl)_2^+$ rereduction), both rates exhibit a similar dependence on pH. A similar correspondence between phase III and ferricytochrome $c_1 + c_2$ reduction has been shown to be the case under optimal conditions when Q_ZH_2 is present [7].

Proton-binding kinetics and magnitudes at high potentials

At E_h values above 300 mV the kinetics of slow carotenoid phase III also coincide with the kinetics of antimycin-sensitive proton binding (H_{II}^+), which in the high-E_h range can also be observed in the presence of valinomycin [13]. Proton-binding kinetics, measured both in the presence and absence of antimycin, and the difference between the two, are shown in Fig. 4D–F. These are compared to the carotenoid band shift kinetics (Fig. 4A–C) and the $(BChl)_2^+$ rereduction kinetics (Fig. 4G–J). Clearly, the kinetics of the antimycin-sensitive portion of each (Fig. 4C, F, I and J) are quite similar. This similarity is surprising in light of the fact that the carotenoid band shift cannot be observed in the presence of valinomycin, yet H_{II}^+ binding is not measurably apparent in the absence of valinomycin. Fig. 4I and J suggests that valinomycin has no effect on the antimycin-sensitive electron transfer,

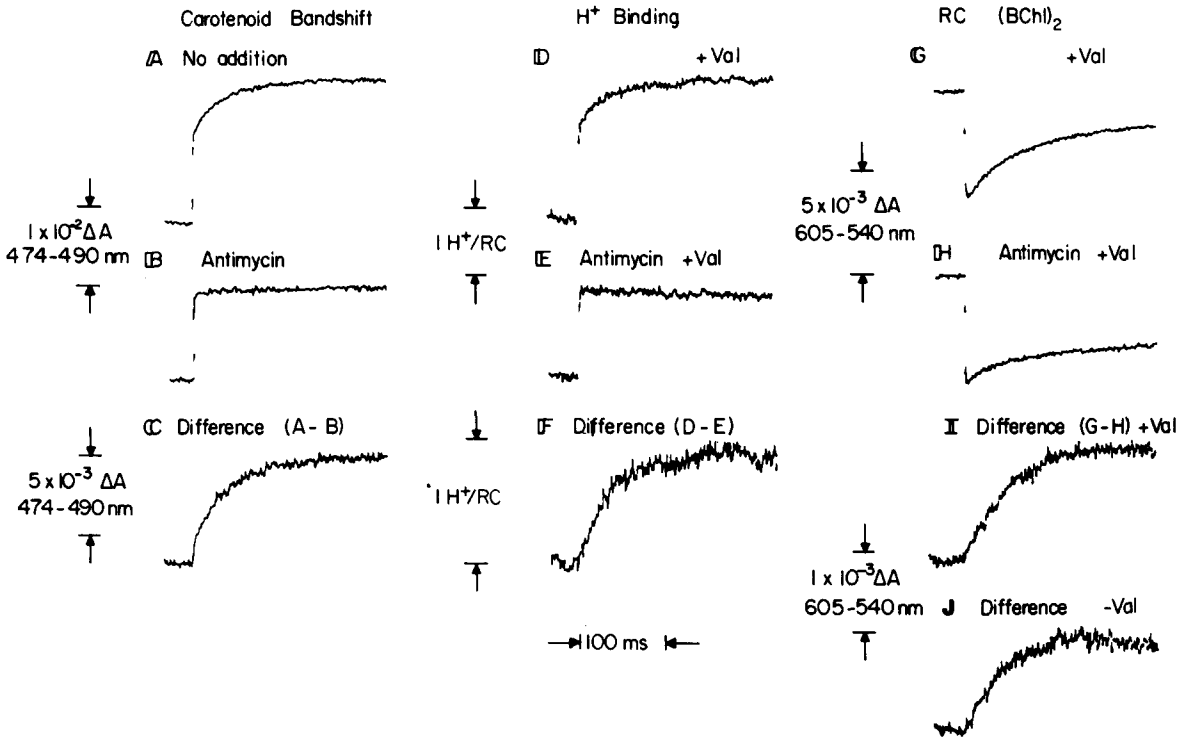

Fig. 4. The kinetic correlation of antimycin-sensitive carotenoid band shift, H^+ binding and reaction center (RC) $(BChl)_2$ reduction. Chromatophores (150 nM reaction center) were suspended in 100 mM KCl, 1 mM $MgCl_2$, 50 μM chlorophenol red, pH 6.0, $E_h = 380$ mV. 1 μM antimycin and 2 μM valinomycin were added where indicated.

so we conclude that at $E_h > 300$ mV, and despite the puzzling finding that the two cannot be seen together, the electrogenic step and the H_{II}^+ binding step share a common rate-limiting step.

The E_h dependency of the attenuation of the slow antimycin-sensitive carotenoid band shift at higher redox potentials

We have noted that the slow phase of the carotenoid band shift does not follow the Nernst curve of $(BChl)_2$ oxidation-reduction at 450 mV (as does phase I). It attenuates over a range that is lower than that expected. The apparent E_m values of the course of attenuation are somewhat pH dependent: pH 6.0, 390 mV; pH 7.5, 360 mV; pH 9.4, 320 mV (see Fig. 2).

Discussion

We have examined flash-activated electron-transfer and electrogenic reactions in ubiquinol-cytochrome *c* oxidoreductase when Q_Z is oxidized. We find under these conditions that a significant fraction of the reducing equivalents makes a complete passage through the ubiquinol-cytochrome *c* oxidoreductase; this is seen as antimycin-sensitive reduction of flash-oxidized cytochrome $c_1 + c_2$ or $(BChl)_2$. The passage of reducing equivalents through the oxidoreductase is coupled to an electrogenic reaction as indicated by an antimycin-sensitive phase of the carotenoid band shift. However, the rate of passage of reducing equivalents through the ubiquinol-cytochrome *c* oxidoreductase is about 10–20-fold slower when Q_Z is oxidized compared to that observed when Q_Z is reduced (Q_ZH_2) before activation.

The reasons for the slower rates observed when Q_Z is present can be considered in two classes of electron-transfer models:

Class 1: Models which are based on more traditional linear redox loop chemiosmotic schemes [1] fall into class 1. In such schemes under optimal conditions with Q_ZH_2 present before flash activation, the redox couple closely associated with the

electrogenic reaction is considered to be $Q_ZH_2/Q_Z^-(H^+)$ [1]. In contrast, starting with Q_Z initially oxidized, the redox couple involved could now be $Q_Z^-(H^+)/Q_Z$. Thus, the rate differences observed in the electrogenic reaction and in the rereduction of cytochrome $c_1 + c_2$ may be an expression of the different quinone couples involved, and their different energetic and kinetic properties.

Class 2: Models utilizing the principal element of the Q-cycle, the 'oxidant-induced reduction' sequence, fall into class 2. Q_ZH_2 is required in this class of model to complete the electron-transfer sequence that promotes the electrogenic reaction. Thus, if Q_ZH_2 is present at the time of activation then electron-transfer and coupled reactions occur promptly. However, if oxidized Q_Z is present the system must first generate Q_ZH_2 by delivery from some source of two reducing equivalents to the Q_Z of each ubiquinol-cytochrome *c* oxidoreductase; hence, the system could need more time to complete the electron-transfer cycle. *

How the two-electron reduction of Q_Z can happen after single-turnover activation can be envisaged as follows: (a) by the donation of reducing equivalents to one ubiquinol-cytochrome *c* oxidoreductase from two different reaction centers; and/or (b) by transfer of reducing equivalents between ubiquinol-cytochrome *c* oxidoreductases that have been singly reduced by one reaction center to yield one population of oxidoreductases with Q_Z and the other with the required Q_ZH_2. It is clear that suggestions such as these contain possible reasons for the pronounced heterogeneity that we have observed in the kinetics. This is discussed in detail in the next section.

The fractional passage of reducing equivalents through the ubiquinol-cytochrome c oxidoreductase when oxidized Q_Z is present

The electron transfer through the ubiquinol-cytochrome *c* oxidoreductase when oxidized Q_Z is present is characterized not only by the slower rate but also by the smaller magnitude of the electron transfer in a time that matches electrogenic events. It is clear from Fig. 1 and from Table I that ferricytochrome $c_1 + c_2$ (experiment done at E_h 200–220 mV) or $(BChl)_2^+$ (experiment done at E_h 350–380 mV) become only partly reduced in 100 ms; in each case the remainder is reduced very much more slowly. Three probable sources can contribute to this biphasic behavior.

(a) Biphasic kinetics due to energetic limitations. In the coupling of electron transfer to the electrogenic reaction, it is possible that there is insufficient free energy available in the redox reactions with respect to the prevailing electric potentials generated to permit a complete reduction of ferricytochrome $c_1 + c_2$. This effect is clear at E_h 100 mV (Q_ZH_2 present) and at 220 mV (oxidized Q_Z and ferrocytochrome $c_1 + c_2$ present) as shown by the stimulated reduction upon addition of valinomycin (K^+) (Table I). Thus, the uncoupling by valinomycin of electron transfer from the generation of significant membrane potential removes the energetic feedback and promotes a more complete rereduction of cytochrome $c_1 + c_2$. In contrast, at E_h 350 mV (oxidized Q_Z and ferricytochrome $c_1 + c_2$ present), this source appears to be unimportant; under these conditions $(BChl)_2^+$ rereduction kinetics are virtually unaltered by the addition of valinomycin (Fig. 4 and Table I). A possible reason for this insensitivity to valinomycin at E_h 350 mV is that the membrane potential formed by the rapid phase at E_h 350–380 mV is much less than that encountered at $E_h < 220$ mV and may be insufficient to prevent electron transfer energeti-

TABLE I

THE FRACTIONS OF $(BChl)_2^+$ OR FERRICYTOCHROME $c_1 + c_2$ REREDUCTION AFTER A SINGLE-TURNOVER FLASH

The values were obtained from kinetic traces similar to those in Fig. 1. The extent of antimycin-sensitive rereduction of $(BChl)_2^+$ (experiment done at E_h 350 mV, measured 100 ms after flash activation) or ferricytochrome $c_1 + c_2$ (E_h 220 mV, measured at 100 ms and at E_h 100 mV, measured 10 ms after the flash) was divided by the extent of oxidation in the presence of antimycin.

E_h (mV) (pH 7.0)	No addition	+ valinomycin
350	0.35	0.37
220	0.41	0.81
100	0.69	0.97

* It is also worth mentioning, however, that class 2 models are not restricted to Q-cycle schemes; various modified linear schemes can be classified similarly if the formation of Q_ZH_2 is mandatory. More work on these matters is needed.

cally. However, an equally important contribution to the valinomycin insensitivity at E_h 350 mV may be because the free energy difference (ΔE_h) between the reductant and oxidant of the ubiquinol-cytochrome *c* oxidoreductase is expected to be larger at 350 mV than that at below 300 mV after single-flash activation.

(b) Biphasic kinetics arising from the presence of Q_Z. A second source of the biphasic behavior may be an important expression of the way the system succeeds in useful, energy-coupled electron transfer through the ubiquinol-cytochrome *c* oxidoreductase when Q_Z is oxidized. Thus, under optimal conditions when Q_ZH_2 is present (E_h 100 mV), little biphasic behavior is observed in the presence of valinomycin. In contrast, when oxidized Q_Z is present, biphasic behavior is apparent even in the presence of valinomycin; in experiments done at 220 and 350 mV, about 20 and 50% of rereduction of cytochrome $c_1 + c_2$ and $(BChl)_2^+$, respectively, is very slow (Table I). Corresponding to this very slow phase of reduction, therefore, we should expect some reducing equivalents to be stranded at the low-potential end of the system for hundreds of milliseconds. Indeed, O'Keefe and Dutton [8] have already reported that about 30% of the cytochrome *b*-560 population stays reduced for more than 100 ms at both E_h 220 and 380 mV.

While there seems to be no simple explanation to account for the stranded electrons by simple linear schemes of class 1, the models of class 2 described above, which include elements of the Q-cycle, can readily account for these data. In these models we must presume that two reducing equivalents are delivered to one ubiquinol-cytochrome *c* oxidoreductase as discussed in the previous section; however, in the population of ubiquinol-cytochrome *c* oxidoreductases that have genereated a Q_ZH_2, only one of the electrons will appear at the oxidizing end because the oxidant-induced-reduction element will put the other on a low-potential component such as cytochrome *b*-560 at the reducing end. It is conceivable via lateral interaction between ubiquinol-cytochrome *c* oxidoreductases that the 'stranded' electron may come together with another stranded electron from another ubiquinol-cytochrome *c* oxidoreductase to form Q_ZH_2 again in half the population and to proceed with the same reaction. Since we see a significant fraction of stranded electrons for a long period, this kind of lateral interaction must be slow or restricted to a small fraction of the ubiquinol-cytochrome *c* oxidoreductase population. This point warrants further investigation from the standpoint of class 2 mechanisms and the question of lateral mobility.

(c) A further source of heterogeneity. Finally, we recognize another complication that may be attributed to one aspect of the nature of the interaction between reaction center and ubiquinol-cytochrome *c* oxidoreductase, and is relevant to the results presented here. Recently, O'Keefe et al. [14] recognized that at E_h values between 300 and 400 mV, about 40% of the reaction center population appears not to interact on a redox basis with the ubiquinol-cytochrome *c* oxidoreductase for at least 10 s; within this time the reaction center behaves as though isolated and, following a single-turnover activation, an electron in this population is seen to be stranded on Q_B (also called Q_{II}, the secondary quinone of the reaction center) and does not enter the ubiquinol-cytochrome *c* oxidoreductase. No such population is detectable in experiments done below E_h 300 mV. Thus, for this reason, in our experiments, we should expect that measurements done at 300–400 mV will display extra stranded reducing equivalents when compared to measurements done at 200–250 mV. This expectation is borne out as shown in Table I. Compared to the experiments at 220 mV, the experiment at 350 mV (valinomycin present) shows a higher fraction of reducing equivalents stranded long after the $t_{1/2}$ 20 ms phase is complete. In other words, at 350 mV with fewer reducing equivalents available to generate Q_ZH_2 to permit passage through the ubiquinol-cytochrome *c* oxidoreductase, a smaller number emerges at the high-potential end to rereduce the $(BChl)_2^+$.

Correlation of electron transfer through the ubiquinol-cytochrome c oxidoreductase with the magnitude of carotenoid band shift phase III

When oxidized Q_Z is present the magnitude of the carotenoid band shift phase III is smaller than under the optimal conditions when Q_ZH_2 is present. Table II summarizes the proportions of phases I–III observed at 220 and 350 mV and compares them with those measured at 100 mV when Q_ZH_2

TABLE II

EXTENT OF CAROTENOID BAND SHIFT PHASE III AND CORRELATION WITH ELECTRON TRANSFER THROUGH THE UBIQUINOL-CYTOCHROME *c* OXIDOREDUCTASE

In A, the extent of antimycin-sensitive carotenoid band shift (phase III) was divided by the extent after the addition of antimycin (phase I + II) at E_h 220 mV or at E_h 100 mV. At E_h 350 mV, the antimycin-insensitive phase does not include most of phase II (which corresponds the electron transfer from cytochrome c_2 to $(BChl)_2^+$) and the antimycin-sensitive phase includes phases III and II because all electrons coming through ubiquinol-cytochrome *c* oxidoreductase after generating phase III then are available to generate phase II. Therefore, the divided value at E_h 350 mV, antimycin-sensitive phase/antimycin-insensitive, which was 0.64, is not suitable for comparison with the values at E_h 220 mV or at E_h 100 mV. The value for phase III/phase I + II was obtained indirectly under the assumption that the extents of phases I, II and III are in the ratio of 0.54 : 0.46 : 1.0 [5,23] when the same number of electrons were transferred for each phase and that 0.16 ferrocytochrome c_2 per reaction center is present at E_h 350 mV (pH 7.0) (0.5 cytochrome c_2 (with $E_{m,7}$ 330 mV) per reaction center is functional in the submillisecond time scale; Matsuura, K., Marrs, B. and Dutton, P.L., unpublished observations). The calculations were as follows: estimated phase III = [antimycin-sensitive phase (0.64)]×[1.0/(0.46 + 1.0)]; estimated normalizing factor, phase I + II = [antimycin-insensitive phase (1.0)]/(0.16×0.46 + 0.54). In B, the extent of the antimycin-sensitive rereduction of $(BChl)_2^+$ (measured at E_h 350 mV) or cytochrome $c_1 + c_2$ (measured at E_h 220 and 100 mV) was divided by the maximum extent of oxidation that is seen in the presence of antimycin. Since cytochrome $c_1 + c_2$ is in rapid equilibrium with the Rieske FeS center, the values which include the Rieske FeS center are estimated and shown in parentheses. The assumptions used are that the $E_{m,7}$ values of the Rieske FeS center, cytochrome c_1 and cytochrome c_2 are 280, 290 and 330 mV, respectively (Matsuura, K., Marrs, B. and Dutton, P.L., unpublished observations). The $E_{m,7}$ value of cytochrome c_1 is 30 mV higher than that estimated by Crofts et al. [12] but similar to that reported by Wood [11]. In (A)/(B) Under the assumption that carotenoid band shift phases I + II correspond to one charge movement across the membrane in each reaction center, the phase III per electron ratio reflects the amount of electrogenic charge movement associated with each reducing equivalent transferred through the ubiquinol-cytochrome *c* oxidoreductase. The values in parentheses correspond to those in parentheses in B. Calculations are based on absorbance changes 100 ms after flash activation at E_h 350 and 220 mV and 10 ms after flash activation at E_h 100 mV.

E_h (pH 7.0) (mV)	(A) Carotenoid band shift phase III divided by phase I + II	(B) $(BChl)_2$ or cytochrome $c_1 + c_2$ extent rereduction divided by extent of oxidation	(A)/(B) Phase III per electron transferred through oxidoreductase
350	0.27	0.35	0.77
220	0.47	0.40 (0.35)	1.18 (1.34)
100	0.91	0.71 (0.67)	1.27 (1.36)

is present. Details of the calculations and the assumptions are given in the legend. At 220 mV the observed amplitude of the carotenoid band shift is smaller than that at 100 mV (pH 7.0), consistent with the discussions of the previous section. However, when the smaller extent of ferricytochrome $c_1 + c_2$ reduction is taken into account, and the carotenoid band shift phase III is assessed per electron equivalent emerging from the ubiquinol-cytochrome *c* oxidoreductase, the amplitude of the band shift is similar to that observed at 100 mV when Q_ZH_2 is present. Thus, at 220 mV the magnitude of the electrogenic event per electron usefully negotiating the ubiquinol-cytochrome *c* oxidoreductase is similar to that found under optimal conditions. At 350 mV, however, even after making analogous corrections, the magnitude of carotenoid band shift phase III per electron equivalent returning through the ubiquinol-cytochrome *c* oxidoreductase (presumably via cytochrome c_2) to $(BChl)_2^+$ is smaller. The reason behind this difference is not clear at present; related problems are discussed further after discussing patterns of H^+ binding when oxidized Q_Z is present.

Correlation of proton binding with electron transfer when oxidized Q_Z is present

Pioneering, semiquantitative studies on H^+ binding showed that H^+ was incorporated into chromatophores at a rate and to an extent consonant with them being coupled to redox reactions [15]. It was also clear that in *Rps. sphaeroides* a fraction of the H^+ incorporated occurred in the

millisecond time scale and was antimycin sensitive, appropriate for an association with events in the ubiquinol-cytochrome *c* oxidoreductase [16]. More detailed, quantitative studies [13] confirmed these early works but also indicated that these processes (or the measurements) may be more complicated than that indicated by redox loop mechanisms which include either 'linear' or 'Q-cycle' schemes. The work presented here extends the list of 'anomalous' behavior summarized below:

(1) As shown in Ref. 17, when Q_Z is oxidized but the Rieske FeS center, cytochrome $c_1 + c_2$ and reaction center $(BChl)_2$ are reduced (E_h 250 mV, pH 6.0), a flash elicits an antimycin-sensitive, H^+ binding (designated H^+_{II}) with a half-time that approaches a value 100-fold shorter ($t_{1/2} \approx 0.2$ ms) than any proven electron-transfer reaction of the ubiquinol-cytochrome *c* oxidoreductase that is considered to be involved with antimycin-sensitive H^+ binding to the outside of the membrane. If H^+ binding follows the electrogenic reaction as proposed in both the linear redox loop chemiosmotic scheme and in the Q-cycle scheme, then starting with oxidized Q_Z present, H^+_{II} would be expected to display a half-time approaching 20 ms rather than the 0.2 ms observed.

(2) The p*K* values apparent for the groups responsible for H^+-binding reactions were found to have values of p*K* 8 or less; these were considered unexpectedly low [13]. However, more puzzling was the finding that at pH values well above these p*K* values, where no H^+ binding was detected, cyclic electron transfer was completed without any unusual behavior. Under these high-pH conditions, all phases of the carotenoid band shift were generated normally and, once formed, were stable in the seconds time scale as was the case in Fig. 2. In any chemiosmotic model which includes redox loops, the absence of H^+ movement accompanying an electron transfer back across the membrane following the electrogenic electron transfer would also be electrogenic but in the opposite, counterproductive direction. The carotenoid band shifts formed in phases I–III would therefore be expected to be collapsed subsequent to their formation within the time period required for completion of the cycle; thus, when Q_ZH_2 is present, this collapse would display a $t_{1/2}$ of 1–2 ms which does not occur [13]. A similar discrepancy has been revealed in the work reported here. At E_h 380 mV (pH 6, no valinomycin), there is little or no H^+_{II} binding accompanying or following the generation of carotenoid band shift phase III and completion of the cycle. Thus, within the format of current models, without H^+_{II} translocation the carotenoid band shift phase III would be collapsed following its formation, on a tens of milliseconds timescale. Furthermore, it is equally strange that H^+_{II} binding does in fact occur with the expected $t_{1/2}$ 20 ms kinetics, but only when the electrogenic reaction is collapsed by ion movement artificially promoted by valinomycin K^+.

In the light of these results that seem anomalous with respect to existing chemiosmotic models involving redox loops, it is pertinent to review the possibility that the measurement of H^+ binding is in error. In this regard we would encourage the reader to examine critically the work done on the matter (see Ref. 13). Some points which suggest that the measurements are not the problem are: (a) At $E_h < 300$ mV, the p*K* of H^+_I is 8.0 and the p*K* of H^+_{II} is 7.5. Thus, the failure of flash-activated H^+_{II} binding can be discriminated from the H^+_I binding which survives until higher pH values; (b) at $E_h \approx 350$ mV the failure to measure H^+_{II} binding occurs under conditions where there is successful measurement of H^+_I (see Ref. 13, Fig. 4); (c) work (O'Keefe, D.P., unpublished results) using glass electrodes instead of pH indicator dyes confirms the failure to detect H^+ binding at high pH values. Thus, while there is no room for complacency in this important matter, we can say that currently there is no evidence that suggests that the H^+ measurements fail generally as the pH is raised or that they are erroneously reporting events occurring in the aqueous phase.

Stoichiometries of proton binding at the higher E_h (380 mV, Fig. 4) also display strange behavior if calculated with respect to the number of antimycin-sensitive reducing equivalents that are transported through the ubiquinol-cytochrome *c* oxidoreductase. The number of H^+_{II} binding per reaction center oxidized by a flash is about 0.8 in the presence of valinomycin (Fig. 4). However, if the approx. one-third of the reducing equivalents that come back to $(BChl)^+_2$ represents those that have usefully passed through the ubiquinol-cytochrome *c* oxidoreductase, the H^+_{II}/e^- ratio could

become 2.4! Proton binding that is in excess of the number usually assumed by simple models has been also observed in the lowest E_h range; i.e., when cytochrome *b*-560 is reduced before activation [13]. Under these low-E_h conditions, when calculated for the number of electrons that rereduced photooxidized cytochrome $c_1 + c_2$, H^+_{II} binding per electron is about 3.0 in the presence of valinomycin (recalculated from Fig. 1 of Ref. 13). However, the number of protons bound in the redox potential range in which cytochrome *b*-560 is oxidized and cytochrome $c_1 + c_2$ is reduced (Q_Z or Q_ZH_2 present) before activation seems to be consistent with the $2H^+/e^-$ concept, at least at pH < 6.5. More work is necessary on proton-binding kinetics and stoichiometries.

Other behavior at higher redox potentials

As already mentioned in the last section of Results, the course of attenuation of the slow carotenoid band shifts following single-turnover flash activation delivered to chromatophores at high potentials occurs at lower potentials (about 350–400 mV midpoint) than the Nernst curve of $(BChl)_2/(BChl)_2^+$. This unexpected discrepancy, which occurs in our hands working with intact chromatophores, does not correspond to any known redox source in the chromatophore (see Ref. 14). It does, however, correlate with flash-activated changes of several other functions associated with the chromatophore. These, discussed in terms of what is observed as the E_h is changed to higher values before activation, include: (a) the appearance of damped oscillations of cytochrome *b*-560; (b) the appearance of an absorbance change at 450 nm attributable to a semiquinone oscillating out of phase with the cytochrome *b*-560; and (c) the loss of the 20 ms half-time reduction of $(BChl)_2^+$. Preliminary work (unpublished data) indicates that the slow carotenoid band shift is also to a small extent oscillating at these high potentials, consistent with the earlier discovery by De Grooth et al. [10]. As stated before, the reasons for the change of behavior expressed in these several ways are not yet understood. However, it is clear that this area is highly descriptive and shoud be worth further study.

A proposition that may be relevant to explain the marked variation in electron-transfer patterns observed in chromatophores poised at different redox states

Thus far described, the redox states of the system before flash light activation have a large effect on the patterns of electron transfer through ubiquinol-cytochrome *c* oxidoreductase, the electrogenic reaction and proton binding. The midpoints of the pattern changes center around 150, 300 and 400 mV at pH 7.0 which corresponds to Q_Z, cytochrome $c_1 + c_2$ (or the Rieske FeS cluster) and an unknown source [14], respectively. A possible reason for these pattern changes that is worth considering is that the different patterns of behavior reflect more than simply changing the redox states prior to activation. There is some reason to believe that there is a major alteration in the physical nature of the interaction between the oxidoreductase and reaction center. This could be viewed as follows: (a) When Q_ZH_2 is present, the system functions optimally and all reaction centers, despite their presence in excess over the ubiquinol-cytochrome *c* oxidoreductase [18], appear to operate a ubiquinol-cytochrome *c* oxidoreductase, that is, some oxidoreductases may be operated twice. (b) When oxidized Q_Z is present at E_h < 300 mV, the system retains the ability of all reaction centers interacting with the ubiquinol-cytochrome *c* oxidoreductase, but in this case the source of the behavior change rests mainly on the logistics of delivering two reducing equivalents to reduce Q_Z to the prerequisite Q_ZH_2 in ubiquinol-cytochrome *c* oxidoreductase. (c) Between E_h 300 and 400 mV, it appears that a significant fraction (about 40%) of reaction centers break away from an oxidoreductase and behave as though isolated. Thus, accordingly, this population of reaction centers has a reducing equivalent that is not available for the oxidoreductase after the single activation. (d) At E_h > 400 mV, all reaction centers behave as though isolated. Under these conditions a second flash is necessary to effect cytochrome *b*-560 reduction, and this may happen via a two-electron component such as a member of the quinone pool. We are aware that there may be other explanations for these phenomena; the proposal briefly presented here is intended to be a simplifying framework for discussion and further work.

Speculation on the nature of chemiosomotic proton translocation

The behavior displayed by the proton-binding reactions when compared to electron transfer and to membrane potential generation prompts us to consider other possibilities for the mechanisms of proton translocation, mechanisms that have the facility to accommodate the apparent anomalies. It seems reasonable to consider that proteinaceous proton-binding groups (see Ref. 19) associated with the membrane redox proteins may extend into the protein, not only to constitute some form of transporting device but also to provide a sink for protons. Such an inclusion in the chemiosmotic system can accommodate the anomalies cited for photosynthetic bacteria; similar suggestions have been made arising from studies on chloroplasts [20] and from mitochondria [21,22].

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