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Electron Transfer between Primary and Secondary Donors in *Rhodospirillum rubrum*: Evidence for a Dimeric Association of Reaction Centers†

Pierre Joliot,*,‡ Andrē Verméglio,§ and Anne Joliot‡
Institut de Biologie Physico-Chimique, Paris, France, and SRA C.E.N. Cadarache, Saint-Paul-lez-Durance, France
Received October 12, 1989; Revised Manuscript Received January 4, 1990

ABSTRACT: Light-induced oxidation of the primary electron donor P and of the secondary donor cytochrome c_2 was studied in whole cells of *Rhodospirillum rubrum* in the presence of myxothiazole to slow down their reduction. 1. The primary and secondary electron donors are close to thermodynamic equilibrium during continuous illumination when the rate of the electron transfer is light-limited. This implies a long-range thermodynamic equilibration involving the diffusible cytochrome c_2. A different behavior is observed with *Rhodobacter sphaeroides* R26 whole cells, in which the cytochrome c_2 remains trapped within a supercomplex including reaction centers and the cytochrome b/c complex [Joliot, P., et al. (1989) *Biochim. Biophys. Acta 975*, 336-345]. 2. Under weak flash excitation, the reduction kinetics of the photooxidized primary donor are nearly exponential with a half-time in the hundred microseconds time range. 3. Under strong flash excitation, the reduction of the photooxidized primary donor follows a second-order kinetics. About half of the photooxidized primary donor is reduced in a few milliseconds while the remainder stays oxidized for hundreds of milliseconds despite an excess of secondary donors in their reduced form. The flash intensity dependence of the amplitude of the slow phase of P^+ reduction is proportional to the square of the fraction of reaction centers that have undergone a charge separation. These results are correctly described with a model in which (1) reaction centers are associated in dimers, (2) the affinity of cytochrome c_2 for the reaction center is low when the cytochrome c_2 is reduced but high when oxidized, and (3) electrostatic or steric interactions prevent the binding of two cytochrome c_2 molecules on the same dimer P-P.

Stabilization of the oxidoreduction products after the light-induced charge separation involves subsequent reactions with secondary acceptors and donors. In purple bacteria, two types of *c* cytochromes operate as direct electron donors to the photooxidized primary donor P (Dutton & Prince, 1978). For species such as *Rhodopseudomonas viridis* or *Chromatium vinosum*, the *c* cytochrome contains four hemes and is tightly bound to the reaction center complex, leading to very fast electron donation at room temperature ($t_{1/2} \approx 300$ ns in the case of *Rp. viridis*; Dracheva et al., 1986; Shopes et al., 1987). This reaction remains efficient even at helium temperature (Dutton, 1971; Rutherford et al., 1979; Verméglio et al., 1989; Hubbard & Evans, 1989). In the case of *Rhodobacter* (*Rb.*)[1] *sphaeroides* and *Rhodospirillum* (*Rs.*) *rubrum* species, the photooxidized primary electron donor is reduced by a soluble cytochrome (cyt) c_2 (Dutton & Prince, 1978). This reaction is blocked at temperatures lower than 240 K (Vredenberg & Duysens, 1964). Two phases ($t_{1/2} \approx 3$ μs and 100 μs-1 ms) are observed at room temperature for whole cells (Overfield et al., 1979), chromatophores (Dutton et al., 1975), or purified reaction centers and cyt c_2 of *Rb. sphaeroides* (Dutton & Prince, 1978; Overfield et al., 1979; Overfield & Wraight, 1980). Several lines of evidence indicate that the fast phase is due to cyt c_2 bound to the reaction center (Dutton & Prince, 1978; Overfield et al., 1980), while the effect of the viscosity and ionic strength on the slow phase implies that this process is diffusional (Dutton & Prince, 1978; Overfield et al., 1979; Prince et al., 1974; Van der Wal et al., 1986). We recently showed in *Rb. sphaeroides* R26 whole cells that the diffusion of one molecule of cyt c_2 is restricted to a domain including two reaction centers and one cyt bc_1 complex forming a "supercomplex" (Joliot et al., 1989).

Oxidation of cyt c_2 in *Rs. rubrum* cells is slower by more than 1 order of magnitude than in *Rb. sphaeroides* (Van Grondelle et al., 1976). Measuring the kinetics of cyt c_2 oxidation as a function of the flash intensity or of the viscosity

† P.J. was supported by a contract CEA/Collège de France (no. V.2491.002); A.J. was supported by a contract CEA/CNRS (no. V.2491.001).
* Address correspondence to this author: Institut de Biologie Physico-Chimique, 13, rue Pierre-et-Marie Curie, 75005 Paris (France).
‡ Institut de Biologie Physico-Chimique.
§ SRA C.E.N Cadarache.

[1] Abbreviations: cyt, cytochrome; cyt b/c_1 complex, ubiquinol-cytochrome $c(c_2)$ oxidoreductase; *Rb.*, *Rhodobacter*; *Rs.*, *Rhodospirillum*.

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of the medium, Van Grondelle et al. (1976) and Van der Wal et al. (1986) concluded that the electron transfer from cyt c_2 to the photooxidized reaction center was a pure diffusional process. In experiments performed with purified cyt c_2 and reaction centers from *Rs. rubrum*, the oxidation kinetics were found to be second order and presented no fast phase at any ionic strength (Rickle & Cusanovich, 1979; Van der Wal et al., 1987). This strongly suggests that no tightly bound cyt c_2 is present prior to the charge separation reaction.

Another important difference between *Rs. rubrum* and *Rb. sphaeroides* is that the ratio between the amount of cyt bc_1 complexes and reaction centers lies between 0.5 and 0.7 for the latter (Prince et al., 1978, 1986) while it can be as low as 0.1 for the former (Van der Wal & Van Grondelle, 1983).

These large differences for the electron donation reactions between *Rb. sphaeroides* and *Rs. rubrum* led us to seek a minimal structural organization common to both species.

Materials and Methods

Cells of *Rb. sphaeroides* R26 strain and of *Rs. rubrum* G9 strain were grown in the light in Hutner medium at 30 °C. The so-called "young" or "aged" cells were harvested after 16 or 25–30 h, respectively. For *Rb. sphaeroides* cells, benzoquinone treatment was performed as described by Joliot et al. (1989). This treatment, which inhibits the entry of electrons at the quinone level, allows a precise control of the redox state of the electron donors by addition of KCN, an oxidase inhibitor. In the case of *Rs. rubrum* cells, slight modifications were applied: the bacteria were exposed for 1 min to 0.3 mM benzoquinone, washed twice with 10 mM Tris buffer, pH 8, and then resuspended in the same buffer. We found that, for *Rs. rubrum* cells, a 30 s–1 min continuous light preillumination was necessary to oxidize an endogeneous pool of reductant, which otherwise significantly accelerate the reduction rate of both primary and secondary photooxidized donors. All the experiments but that of Figure 1 were performed with benzoquinone-treated cells.

Spectrophotometric measurements were performed with an apparatus described by Joliot et al. (1980) and improved according to Joliot and Joliot (1984). Actinic excitation was provided either by xenon flashes (3-μs half-time duration) or a 12-V quartz–halogen lamp filtered through a Kodak Wratten 89B film and suitable grey filters to decrease the energy when desired.

The photooxidation of the primary donor P was measured by the difference $\Delta A_{603nm} - 0.66\Delta A_{586nm}$ as described by Joliot et al. (1989) for both bacterial species. The photooxidation of cyt c_t (i.e., cyt c_1 + cyt c_2) was measured as the difference $\Delta A_{551nm} - (\Delta A_{535nm} + \Delta A_{565nm})/2$ in the case of *Rb. sphaeroides* (Joliot et al., 1989). For *Rs. rubrum* cells, due to a small contribution (7%) of absorption changes linked to the photooxidation of the primary donor in this difference, photooxidized cyt c_t was measured as $\Delta A_{551nm} - (\Delta A_{535nm} + \Delta A_{565nm})/2 + 0.07\ (\Delta A_{603nm} - 0.66\Delta A_{586nm})$.

It is worth while pointing out that, in benzoquinone-treated cells in the presence of myxothiazole, we observed no absorption change in the 560–565-nm range characteristic of cyt *b* redox changes.

For Rs. rubrum, the ratio $\epsilon_{c_t}/\epsilon_P$ was calculated by comparing the flash-induced absorption changes occurring between 70 μs and 1 ms for these two electron carriers. This ratio varies from 1.0 to 1.3.

In all experiments but that of Figures 2 and 3, the concentrations of P^+ and cyt c_{t^+} were normalized to the total concentration of reaction centers, measured by the maximal absorption changes induced by continuous illumination.

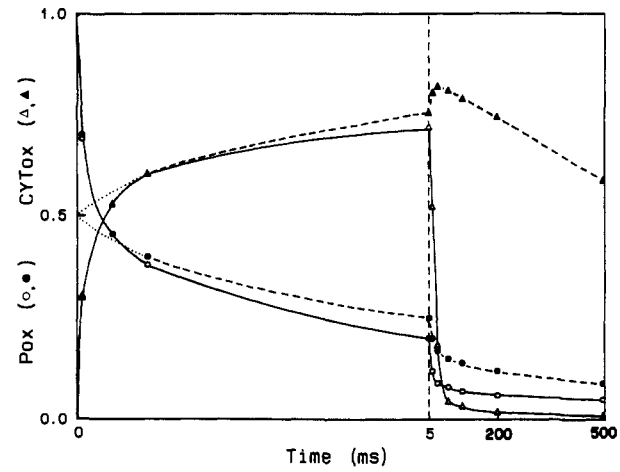

FIGURE 1: Effect of myxothiazole on the time course of P and cyt c_t redox changes induced by a saturating flash. Untreated *Rs. rubrum* young G9 cells; 1 mM KCN. Solid lines, no myxothiazole. Dashed lines, 100 μM myxothiazole. Dotted lines, extrapolation to time zero of the slow phases of P reduction and cyt c_t oxidation.

To estimate the relative contribution of cyt c_2 and cyt c_1 in *Rs. rubrum* cells, the following experiments were performed: we first measured the difference spectrum between cells under anaerobiosis and cells oxidized by addition of 50 mM $K_3Fe(CN)_6$. Bacteria from the same batch were broken by two passages through a French press (16 000 psi) and then centrifuged for 1 h at 200000*g*. The reduced (dithionite) – oxidized [$K_3Fe(CN)_6$] difference spectra of both the supernatant and the pellet were recorded. The comparison of these three difference spectra indicates that the ratio of cyt c_1 to cyt c_2 lies between 0.1 and 0.15. Another indication that cyt c_2 is in large excess compared to cyt c_1 comes from the observation that the absorption changes of the photooxidized cytochrome is centered at 550.5 nm, very close to the cyt c_2 maximum while cyt c_1 peaks at 553 nm (Wynn et al., 1986). This estimation of the amount of cyt b/c_1 complex is in agreement with the one reported by Van der Wal and Van Grondelle (1983).

Results and Discussion

Figure 1 shows the time course of the redox changes of P and cytochrome c_t after a saturating flash given to suspensions of untreated cells of *Rs. rubrum* cells under reducing conditions. The kinetics behavior is multiphasic for both P and cyt c_t absorption changes: ~50% of the photooxidized primary donor is rereduced in less than 1 ms, with a concommitent oxidation of cyt c_t while, as already noticed by Van Grondelle et al. (1976), a significant part of P^+ is stable on a 100-ms time scale although cyt c_t is rereduced with a $t_{1/2} \approx 20$ ms. In the presence of myxothiazole, an inhibitor of electron transfer at the ubiquinol oxidizing site (Meinhardt & Crofts, 1982), the reduction of cyt c_t is strongly inhibited while the matching of the kinetics of the primary donor P and of the cytochrome in the first millisecond is still observed. In benzoquinone-treated cells, the rate of cyt c_t reduction is slowed down (data not shown), which is very likely due to a more oxidized state of the ubiquinone pool. The control experiment shows that, in the presence of KCN and myxothiazole, the same amount of cyt c_t is photooxidized in untreated or benzoquinone-treated cells. We therefore conclude that, in the presence of KCN, cyt c_t is fully reduced in benzoquinone-treated cells.

Oxidation of Primary and Secondary Donors in Different Bacterial Species under Continuous Illumination. As a matter of comparison, the process of oxidation of primary donor P and secondary donors cyt c_t was analyzed in *Rb. sphaeroides*

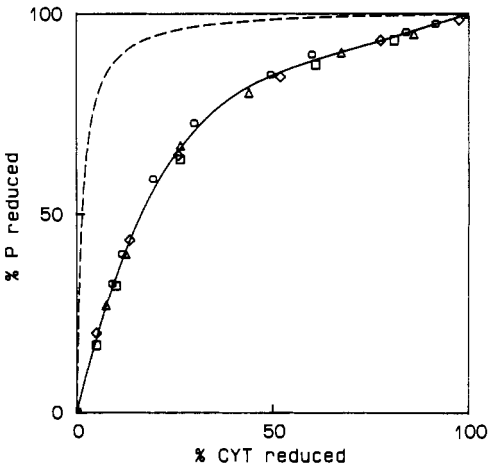

FIGURE 2: Function [P] = f[cyt c_t] for different light intensities. Benzoquinone-treated *Rb. sphaeroides* R26 cells. 40 μM myxothiazole; 2 mM KCN. Solid line: during the course of continuous illumination, I = 100 (○) corresponds to 1$h\nu$/~40 ms per photocenter; 60 (△); 20 (◇); 10 (□). Dashed line: theoretical curve drawn for an equilibrium constant of 70 between P and cyt c_t.

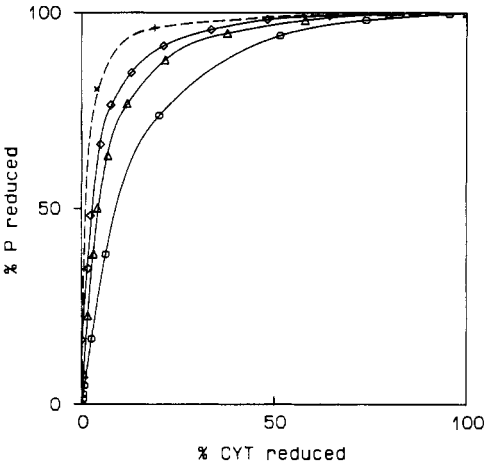

FIGURE 3: Function [P] = f[cyt c_t] for different light intensities. Benzoquinone-treated *Rs. rubrum* G9 cells. 20 μM myxothiazole; 1 mM KCN. Open symbols, continuous illumination: I = 100 (○) corresponds to 1$h\nu$/~40 ms per photocenter; 10 (▲); 2.5 (◇). Reduced P and cyt c_t after a dark period of 5 (×) and 7.5 s (+), following a 0.5-s continuous illumination. Dashed curve: theoretical function, corresponding to a full thermodynamic equilibration between P and cyt c_t (equilibrium constant K = 100).

and *Rs. rubrum*. In both cases, benzoquinone-treated bacteria in the presence of KCN and myxothiazole were submitted to continuous illumination of various intensities. Primary and secondary donors were fully reduced during a 2-min dark adaptation prior to illumination. The functions [P] = f[cyt c_t], computed during the course of illumination, were plotted for *Rb. sphaeroides* (Figure 2) and *Rs. rubrum* (Figure 3). The dashed curves in Figures 2 and 3 show the theoretical functions [P] = f[cyt c_t] corresponding to a full thermodynamic equilibration between P and cyt c_t and deduced from the redox potential values of these carriers. In the case of *Rb. sphaeroides* (Figure 2) and as already discussed by Joliot et al. (1989), primary and secondary donors are far from thermodynamic equilibrium although the relationship between [P] and [cyt c_t] remains essentially independent of the light intensity in a large domain (×10). We interpreted this behavior in terms of "supercomplexes" (Joliot et al., 1989; Lavergne et al., 1989). On the contrary, in the case of *Rs. rubrum* (Figure 3), the experimental functions [P] = f[cyt c_t] are closer to the theoretical one and depend on light intensity. A full thermodynamic equilibration requires several seconds of dark adaptation. This is clearly shown by the redox states of P and cyt c_t measured after a 5 or 7.5-s dark period following a strong continuous illumination since the corresponding experimental points fit the theoretical curve. These results imply that, in the case of *Rs. rubrum*, a long-range thermodynamic equilibration involving a diffusing carrier—very likely cyt c_2—occurs, at least in the 100 ms–few seconds time range. This result implies that, in this time range, thermodynamic equilibration between primary and secondary donors does not require the presence of redox mediators.

Flash-Induced Redox Changes of P and cyt c_t. The time courses of P reduction and of the cyt c_t oxidation were analyzed following a weak or saturating xenon flash. In order to vary the cyt c_t to P ratio, we used bacterial cultures of different ages (see Materials and Methods). This ratio was determined by measuring the total amount of this compound after photooxidation under strong continuous illumination. The ratio cyt c_t/P was about 1.03 and 0.55 in young (Figure 4) and aged cells (Figure 5), respectively. As discussed in Materials and Methods, cyt c_2 appears as the major component of cyt c_t. In the case of aged cultures, we observed that even after excitation by a very weak flash ~5% of the excited reaction centers remain in the P^+ state for several tens of milliseconds. We conclude that ~5% of the reaction centers are not able to react with cyt c_2 and this fraction of the centers has been subtracted in the experiment Figure 5.

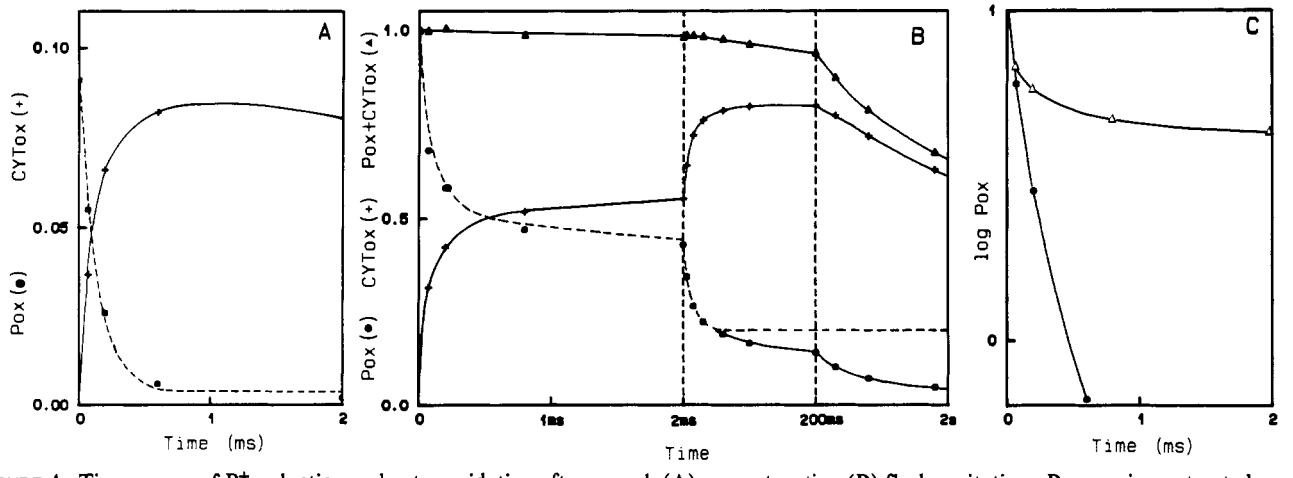

FIGURE 4: Time course of P^+ reduction and cyt c_t oxidation after a weak (A) or a saturating (B) flash excitation. Benzoquinone-treated young G9 cells. 1 mM KCN; 20 μM myxothiazole. Dashed curves: simulation of P^+ reduction with $k_1 = k_2 = 8500$, $k'_2 = k_3 = 90$, $k'_3 = 8500$, and R = 0.8. C: semilogarithmic plot of P^+ reduction. Weak flash (●); saturating flash (△).

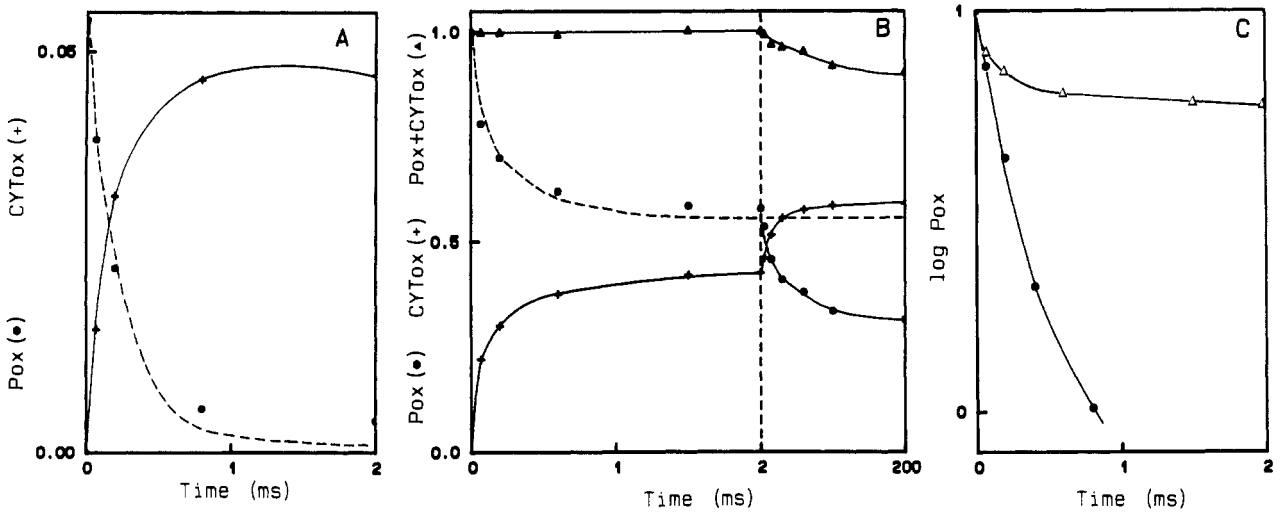

FIGURE 5: Same experiments as in Figure 4, but performed with benzoquinone-treated aged G9 cells. The same kinetics parameters as in Figure 4 were used to compute the simulated functions (dashed lines); $R = 0.45$.

After a weak flash, which hits less than 10% of the centers (Figures 4A and 5A), the reduction kinetics of P reasonably fits an exponential function, as shown by the semilogarithmic plots (Figures 4C and 5C). The half-times of P^+ reduction are ~90 and ~175 μs for young and aged cells, respectively. Thus, the corresponding pseudo-first-order rate constants are roughly proportional to the cyt c_t/P ratio.

After a saturating flash (Figures 4B and 5B), the fast reduction of P^+ approximately follows second-order kinetics, as shown by the semilogarithmic plots (Figures 4C and 5C). A few milliseconds after the flash, a large fraction of P remains in its oxidized form. The total reduction of P^+ is achieved in ~10 s.

In the 0–5-ms time range, the sum of the positive charges present in P and cyt c_t remains constant. This implies that, in this range of time, the number of positive charges included in the FeS protein (not spectrophotometrically detected) is negligible, i.e., that no significant electron transfer occurs between the cyt b/c complex and cyt c_2. Therefore, the millisecond reduction phase of P^+ should be essentially associated with a slow electron-transfer reaction between cyt c_2 and P^+, which explains why myxothiazole does not significantly affect this reduction phase (Figure 1). On a longer time range, the decrease of the sum $[P^+] + [\text{cyt } c_t^+]$ might be associated with a transfer of some positive charges on the FeS protein. Beyond 500 ms, the reduction of both $[P^+]$ and $[\text{cyt } c_t^+]$ might be due to a leak of electrons through the myxothiazole-sensitive ubiquinol site of the cyt b/c_1 complex.

In agreement with Van Grondelle et al. (1976), we conclude from the above experiments that cyt c_2 is a diffusible carrier since (1) the rate of P^+ reduction, as measured after a weak flash excitation, is roughly proportional to cyt c_t concentration, in which cyt c_2 is the major component, and (2) the reduction of P^+ follows a second-order kinetics when the amount of P^+ is of the same order as that of cyt c_2 (saturating flash excitation).

As already reported (Van Grondelle et al., 1976; Rickle & Cusanovich, 1979; Van der Wal et al., 1987), cyt c_2 displays a much lower affinity for the reaction center in *Rs. rubrum* than in *Rb. sphaeroides*, since no microsecond phase could be observed for its oxidation.

An unexpected result is that in the case of young cells, i.e., when $[\text{cyt } c_2] \approx [P]$, the break between the fast and the slow phases of P^+ reduction occurs when 50% of the P's are reduced. The same result is observed in untreated cells, even in the absence of myxothiazole (see Figure 1), and is unchanged for cyt c_t/P ratios ranging 0.7 to 1.1 (data not shown). These results are not explained in models in which isolated reaction centers interact with a diffusible cyt c_2. This point will be discussed later.

It is noteworthy that, in *Rb. sphaeroides*, a comparable break occurs when ~50% of the P^+'s is reduced (Joliot et al., 1989), although the half-times of both fast and slow phases of P^+ reduction are more than 10 times shorter (Dutton et al., 1975; Van Grondelle et al., 1976; Overfield et al., 1979; Joliot et al., 1989). The following structural model, which includes some of the features of the one previously proposed for *Rb. sphaeroides* (Joliot et al., 1989), can account for our experimental results in *Rs. rubrum*: (1) Reaction centers are associated in dimers P–P. This hypothesis has been already proposed by Loach (1976) on the basis of semiquinones dismutations occurring on the acceptor side. However, such a cooperativity has not been confirmed (De Grooth et al., 1978). (2) Each P possesses one site for the binding of cyt c_2. (3) The affinity of reduced cyt c_2 for this site is low but high when oxidized. A "product inhibition" by oxidized cyt c_2 has been already proposed by Moser and Dutton (1988) in experiments performed on purified cyt c_2 and photosynthetic reaction centers of *Rb. sphaeroides*. (4) When one of the sites of the dimer P–P is occupied by cyt c_2^+, the other site is no longer accessible to cyt c_2.

Following a saturating flash excitation, the two P's of the dimer are in their oxidized form. A cyt c_2 molecule reduces one of the P^+'s, which leads to a P^+–P/cyt c_2^+ form. Thus, half of the P^+'s is rapidly reduced and the slow reduction of the remaining fraction will depend on the binding and dissociation rate constants for oxidized and reduced cyt c_2. After a weak flash, most of the P^+'s are included in dimers in a P^+–P state. The reduction of P^+ by cyt c_2 follows a first-order kinetics and leads to fully reduced dimers.

Simulations. According to our model, we have to consider the following reaction center dimer states in which cyt c_2 is quoted as C_2:

P P	P^+ P^+	P^+ P	PC_2^+ P	P^+ C_2^+ P	PC_2^+ P^+	P^+ C_2^+ P
(A)	(B)	(C)	(D)	(E)	(F)	(G)

We omitted the states including a reduced C_2 due to its low affinity constant for the dimer P–P. Moreover, the states C–G can exist under two forms, as C_2^+ can bind to either P of the same dimer P–P.

The rate constants used in the differential equations that

describe the evolution of each of these seven states are defined by the following reactions where P_a and P_a^+ represent the primary donors able to interact with free C_2:

$$P_a^+ + C_2 \xrightarrow{k_1} P\,C_2^+ \quad (1)$$

$$P_a + C_2^+ \underset{k'_2}{\overset{k_2}{\rightleftharpoons}} P\,C_2^+ \quad (2)$$

$$P_a^+ + C_2^+ \underset{k'_3}{\overset{k_3}{\rightleftharpoons}} P^+\,C_2^+ \quad (3)$$

With

$$[P_a^+] = 2[B] + [C] \text{ and } [P_a] = 2[A] + [C]$$

This model does not take into account electron-transfer reactions between cyt c_2 and cyt c_1, which probably occur on a long time range (>5 ms).

A computer program was developed by J. Lavergne to simulate the time course of P^+ reduction and cyt c_2 oxidation following a flash excitation. The theoretical curves corresponding to P^+ reduction after nonsaturating or saturating flash excitation are drawn in Figure 4A,B for young cells and Figure 5A,B for aged cells (dashed curves). A good fit is obtained by using the same set of rate constants for both young and aged cells. Only the ratio R = [cyt c_2]/[P] depends upon the age of the culture and is equal to 0.80 and 0.45 for young and aged cells, respectively. Experimentally, we found that the ratio R' = [cyt c_t]/[P] equal to 1.04 and 0.57 for young and aged cells, respectively. Therefore, cyt c_1 would represent ~0.2 cyt c_t, a value close to the one biochemically determined [see Materials and Methods and Van der Wal and Van Grondelle (1983)].

For both young and aged cultures, the kinetics of the fast phase depends upon the product k_1R, which determines the value of the pseudo-first-order constant measured under weak flash excitation.

In the case of young cultures, i.e., [cyt c_2] > 0.5[P], the kinetics of the slow phase depends upon the values of k_2 and k'_2 but are rather independent of the values of k_3 and k'_3. The fit between experimental and theoretical curves remains excellent up to 50 ms, which suggests that in this particular batch (Figure 4), no electron transfer between the cyt b/c complex (FeS–cyt c_1) and cyt c_2 occurs during this time range.

In the case of aged cultures, i.e., [cyt c_2] < 0.5[P], the amplitude of the fast phase is limited by the concentration of cyt c_2. The slow phase of P^+ reduction and cyt c_t oxidation observed in the 2–100-ms range is very likely due to electron transfer from cyt c_1 to cyt c_2. As expected, a significant decrease of the sum $[P^+]$ + [cyt c_t^+] is observed in this time range. Thus, electron transfer from FeS to cyt c_t appears significantly faster in the experiment shown in Figure 5 than in Figure 4. We have no interpretation for the difference observed between aged and young cultures concerning the rate of electron transfer between the cyt b/c complex and cyt c_2. One possibility would be that in aged cells, the relative amount of cyt c_1 versus cyt c_2 is higher than in young cells.

Time Course of P and cyt c_2 Redox Changes as a Function of the Flash Energy. A powerful way to test the validity of our model is to measure the relative amplitude of the fast and the slow phases of P^+ reduction as a function of the flash energy. The relative concentrations of the states P^+–P^+ and P^+–P induced by a flash excitation depend upon the fraction F of photocenters that underwent a charge separation: $[P^+P^+] = F^2$ and $[P^+P] = 2F(1 - F)$. After completion of the fast phase, these two states would lead to states P^+–P C_2^+ and P–P C_2^+, respectively. Therefore, in this condition $[P^+] = 0.5F^2$,

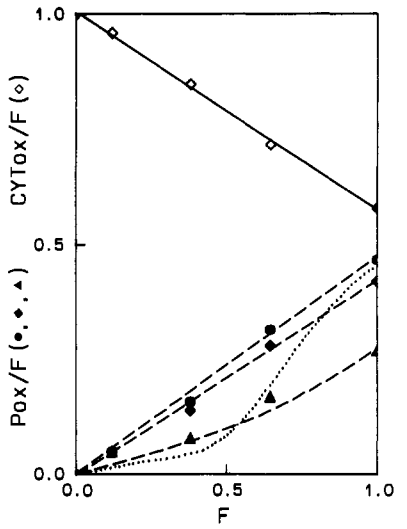

FIGURE 6: Fraction of oxidized P and cyt c_t as a function of the flash energy. Benzoquinone-treated young G9 cells. 1 mM KCN; 20 μM myxothiazole. F, fraction of photocenters hit by the flash. Fraction of oxidized P measured 1.2 (◆) and 5 ms (▲) after the flash. Linear extrapolation to time zero of the slow reduction of P^+ (●). Dashed curves: simulated curves; see parameters in the text. Dotted curve: simulated curve, assuming isolated reaction centers and [cyt c_t] = 0.55[P]. Fraction of oxidized cyt c_t measured 1.2 ms after the flash (◇).

i.e., $[P^+]/F = 0.5F$. In an experiment (Figure 6) performed with young cultures, the ratio $[P^+]/F$ measured 1.2 and 5 ms after the flash, i.e., when the fast phase is completed, is plotted as a function of F. When the slow phase of P^+ reduction is extrapolated to time zero, the corresponding points are close to the function $[P^+]/F = 0.5F$, as expected from the proposed model. From a more refined analysis using Lavergne's computer program we draw the theoretical functions at 1.2 and 5 ms (dashed curves). An excellent fit is obtained for the following set of parameters: $k_1 = k_2 = 15\,000$, $k'_2 = k_3 = 200$, $k'_3 = 15\,000$, and $R = 0.9$. For these cells, the value of k_1 is 1.8 times higher than that of the experiment in Figure 4. Note that k_1 is proportional to the density of photocenters within the photosynthetic membrane. Thus, a variability of this density could explain the different values of k_1 observed in these two batches.

In Figure 7 the same experiments were performed on aged cultures (cyt c_t/P ratio of 0.55), i.e., under conditions in which the amplitude of the fast phase of P^+ reduction after a saturating flash is limited by the concentration of cyt c_2. The function $[P^+]/F = f(F)$ is correctly fitted when using the following set of parameters: $k_1 = k_2 = 9000$, $k'_2 = k_3 = 100$, $k'_3 = 9000$, and $R = 0.39$.

Effect of the Redox Poise on the Time Course of P^+ Reduction after a Nonsaturating Flash Excitation. In the experiment shown in Figure 8, the cells are dark-adapted for 2 min in the absence or in the presence of 2 mM KCN. As already discussed, P and cyt c_t are fully reduced in the presence of KCN, which inhibits the oxidases. In the absence of KCN, more oxidizing conditions are established and ~54% of the cyt c_t is in its oxidized form while P is mainly reduced. Bacterial cells were then submitted to a weak flash, which hit ~5% of the reaction centers, leading to an amount of positive charges much smaller than the amount of reduced cyt c_2 whatever the redox conditions. In contrast to the monophasic reduction of P^+ already observed under reducing conditions, a biphasic process is observed under oxidizing conditions. This biphasicity is not due to the unavailability of reduced cyt c_2 since, under saturating flash excitation, a large amount of cyt c_2 can be rapidly photooxidized. On the other hand, due to

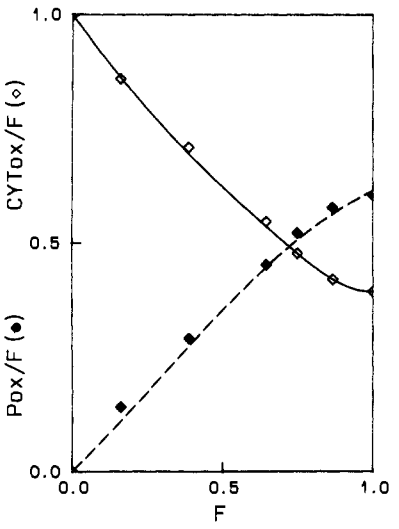

FIGURE 7: Same experiments as in Figure 6, but performed with benzoquinone-treated aged G9 cells. Fraction of oxidized P (◆) and oxidized cyt c_t (◇) measured 1.2 ms after the flash. Dashed curve: simulated curve; see parameters in the text.

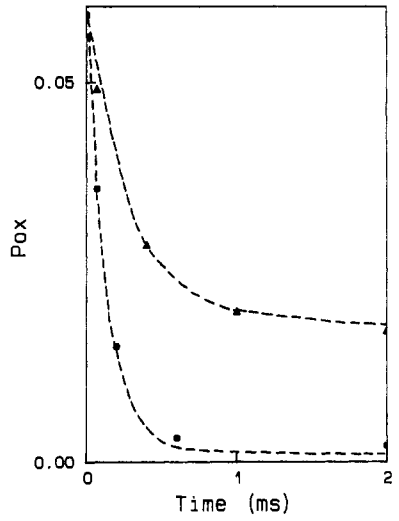

FIGURE 8: Time course of P^+ reduction after a nonsaturating flash excitation for different redox poises in benzoquinone-treated G9 cells. The flash hits 5.5% of the photocenters. No KCN (▲); 2 mM KCN (●). Dashed curves: simulated functions; see parameters in the text.

the high affinity of oxidized cyt c_2 for reduced P ($k_2/k'_2 \approx 85$), a major fraction of the dimer should be in state D (P–P C_2^+) prior to the flash excitation. A weak flash will induce equal concentrations of states E (P–P^+ C_2^+) and F (P^+–P C_2^+). P^+ belonging to state F is slowly reduced due to the high affinity constant of C_2^+ for reduced P; consequently, rapidly reduced P^+ should belong to state E, which implies a rapid dissociation of C_2^+ from P^+. A good fit of the experimental data is obtained for both redox conditions with $k_1 = k_2 = 8500$, $k'_2 = k_3 = 100$, $k'_3 = 8500$, and $R = 0.85$ and assuming that 40% of the cyt c_2 is in the oxidized form. Any values of $k'_3 \geq k_1$ and $k'_3/k_3 \geq 80$ lead to a fit of equivalent quality.

Alternative Models We examined if alternative models in which reaction centers are isolated could satisfactorily represent our experimental results.

Model 1. The two-phase kinetics of P^+ reduction observed after a saturating flash can be interpreted by assuming that the concentration of cyt c_2 never exceeds about half of the concentration of the reaction centers. This hypothesis is unlikely, at least in young cells in which the biochemical analysis shows that the concentration of cyt c_2 is ~0.8 times the concentration of the reaction centers. Nevertheless, we further tested this hypothesis in the experiment of Figure 6, assuming [cyt c_2] = 0.55[P] and an equilibrium constant of 100 for the electron transfer from cyt c_2 to P. The deviation between the theoretical curve (Figure 6, dotted curve) and the experimental data clearly eliminates this model.

Model 2. We have examined an hypothesis similar to the "product inhibition" hypothesis proposed by Moser and Dutton (1988), in which cyt c_2^+ has a large affinity for reaction centers in P^+ state. This model correctly describes the two-phase kinetics of P^+ reduction by assuming a high rate constant for the fixation of cyt c_2^+ on P^+ and a large affinity constant. However, this model fails to describe the kinetics observed after a nonsaturating flash excitation. The predicted amplitude of the slow phase after a nonsaturating flash excitation remains much larger than the one experimentally measured. This model also failed to predict P^+ reduction kinetics when one varies the redox poise.

Conclusion

In our model, the binding of cyt c_2^+ to one of the two sites of the dimer P–P renders the second site totally inaccessible. This large anticooperativity suggests that the distance between the binding site of cyt c_2 belonging to each of the reaction centers is small. Therefore, the two reaction centers are very likely in direct interaction, while the core antenna should be localized to the dimer P–P periphery. The occurrence of structures including several reaction centers and their associated antennas has been directly established by singlet–singlet annihilation measurements (Vos et al., 1986). Association between two reaction centers into "duplex" has been proposed by Loach (1976) for *Rs. rubrum* and by Snozzi and Crofts (1985) for *Rb. sphaeroides* chromatophores. In this last study, the dimeric association of reaction centers appears to be stabilized by cyt c_2. In the case of *Rs. rubrum*, we conclude that cyt c_2 is not essential for the formation of reaction centers dimers, considering the low affinity of reduced cyt c_2 for the reaction center.

It is worth noting that the "product inhibition" by cyt c_2^+ is of little functional consequence when bacteria are submitted to a continuous illumination, since the affinity constant is much smaller for state P^+ C_2^+ than for state P C_2^+. Consequently, the released cyt c_2^+ could be rereduced by a subsequent reaction with the cyt b/c_1 complex. On the contrary, in the case of model 2 proposed above, we predict that under strong continuous illumination, most of the reaction centers will be blocked in the P^+ C_2^+ state.

According to our model, the affinity of cyt c_2 for P strongly depends on their redox states. For instance, the absence of a microsecond phase in P^+ reduction implies that the affinity constant of reduced cyt c_2 for P is lower than 0.05, while the affinity of oxidized cyt c_2 for P is ~100. Therefore, redox changes of cyt c_2 and P induce a variation of at least a factor of 2000 in their affinity constants. Changes in electrostatic interactions corresponding to the different redox states of cyt c_2 can only partially account for this very large variation of the equilibrium constant.

Acknowledgments

We are debtful to J. Lavergne, who developed the theoretical analysis of the different kinetics models proposed in this paper.

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