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Evidence for supercomplexes between reaction centers, cytochrome c_2 and cytochrome bc_1 complex in *Rhodobacter sphaeroides* whole cells

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Light-induced oxidation of the primary electron donor P, the secondary electron donors cytochrome c_2, cytochrome c_1 and the FeS Rieske protein was studied in whole cells of *Rhodobacter sphaeroides* in the presence of myxothiazol which slows down their reduction. During continuous illumination, the primary and secondary donors are not in thermodynamic equilibrium, despite the rate of electron transfer being light-limited. After a short saturating flash excitation, part of the photooxidized P is not rereduced by the secondary electron donors, although more than 50% of them are still in the reduced state. These results are interpreted in terms of a model where reaction centers, cytochrome c_2 and cytochrome bc_1 complexes form supercomplexes. About 70% of these supercomplexes include two reaction centers, one cytochrome c_2 and one cytochrome bc_1 complex, while the remaining ones include only two reaction centers and one cytochrome c_2. During the course of illumination, the redox equilibrium is achieved only within each supercomplex, considered as an isolated entity. On the other hand, equilibration between redox components included in different supercomplexes occurs slowly in the dark via diffusing electron carriers. Our model also readily explains the biphasic kinetics of reduction of the primary donor and of the oxidation of the secondary donor. According to our hypothesis, only half of the reaction centers is tightly associated with cytochrome c_2. Following a saturating flash excitation, in this fraction of the reaction centers the reduction of the primary donor P^+ is completed in less than 25 μs. The reduction of the remaining P^+ implies two sequential reactions: (1) rereduction of cytochrome c_2 by cytochrome c_1 ($t_{1/2} \approx 130$ μs); and (2) association of this reduced cytochrome c_2 with P^+ ($t_{1/2} \approx 60$ μs).

Introduction

The early step of the transformation of light energy into chemical energy in purple bacteria is a cyclic electron transfer linked to the transport of protons across the intracytoplasmic membrane. This process involves two membrane-bound multisubunit complexes, the photosynthetic reaction centers and the cytochrome bc_1 complexes.

The stoichiometry between these two types of complexes varies according to the species considered. In *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, estimation lies between 0.5 and 0.7 cytochrome bc_1 complex per reaction center depending on the extinction coefficients used [1–3]. This ratio can reach the value of 2 for *Rb. capsulatus* cells grown in high light intensity [4]. On the other hand, *Rhodosprillum rubrum* contains as low as 0.1–0.2 cytochrome bc_1 complex per reaction center [5].

The thermodynamic and kinetic properties of the

Abbreviations: *Rb, Rhodobacter*; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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different electron-transferring components have been intensively studied by potentiometric methods and by flash spectroscopy [6,7]. The primary charge separation corresponds to the transfer of one electron from a high-potential donor P ($E_{m7} = 450$ mV) to a low-potential acceptor Q_A ($E_{m7} = -180$ mV) and subsequently to the secondary acceptor Q_B. The photooxidized primary electron donor P is rereduced by *c*-type cytochromes with a biphasic kinetics [8–10]. The fast phase ($t_{1/2} \approx 3$ μs) is linked to the oxidation of the soluble cytochrome c_2 ($E_{m7} = 345$ mV) and the slower phase ($t_{1/2} \approx 100$ μs–1 ms) to the oxidation of the cytochrome c_1 ($E_{m7} = 265$ mV), presumably via cytochrome c_2 [11]. The electron donor to cytochrome c_1, the Rieske-type protein (denoted FeS), is oxidized with a half-time of 200 μs following a lag-time of 100 μs [12,13]. This behaviour is consistent with the difference in redox potential between the cytochrome c_1 (265 mV) and the FeS center ($E_{m7} = 290$ mV). The cytochromes $c_2 + c_1$ and the oxidized FeS center are then reduced by a quinone molecule, Q_z, with second order kinetics [3]. This reaction is inhibited by addition of myxothiazol, an antibiotic inhibitor [13].

Although freely mobile components, ubiquinol in the hydrophobic domain of the membrane and soluble cytochrome c_2 in the periplasmic space, appear to connect the reaction centers to the cytochrome bc_1 complexes, several lines of evidence suggest that super complexes between reaction centers, cytochrome c_2 and cytochrome bc_1 may exist.

The simplest supercomplex results from the association between purified reaction centers and cytochrome c_2 or *c*. Several investigators [8,10,14–19] reported a cytochrome photooxidation with two phases of equal amplitude and rate constants of approx. 1.10^6 and 2.10^3 s^{-1}, respectively. This behavior has been explained in terms of a model where the cytochrome is bound to the reaction center is favorable ('proximal') or unfavorable ('distal') state for electron transfer [15–19]. In *Rb. sphaeroides* chromatophores, Prince et al. [3] showed, by determining the extent of both photooxidizable P and cytochrome as a function of the flash intensity, that the reduced cytochrome is unable to move between reaction centers.

For the *Rhodospirillum rubrum* species, Loach [20] developed the so-called 'duplex model' in which the two semiquinones formed by a single flash excitation can cooperate to form a quinol molecule. No evidence has been reported to date for such a cooperation in other species of photosynthetic bacteria. Snozzi and Crofts [21] also proposed a duplex model in which the association between two reaction centers is stabilized by cytochrome c_2 in *Rb. capsulatus* chromatophores. From a detailed study of the photoreduction of cytochrome *b*-560 at high redox potential, O'Keefe et al. [22] proposed that part of the reaction centers are bound to a cytochrome bc_1 complex, at least on a time scale of 5 s. More recently, Prince et al. [1] showed that fast electron transfer occurs between photooxidized reaction center and cytochrome bc_1 complexes in a strain of *Rb. capsulatus* depleted in the gene of cytochrome c_2. This demonstrates that direct interaction between the two complexes is effective, at least in that species.

On the other hand, Van Grondelle et al. [23] and Van der Wal et al. [5] concluded from experiments where the kinetics of cytochrome c_2 was followed as a function of the flash intensity or of the viscosity of the medium, that electron transfer from reaction center to cytochrome c_2 in *R. rubrum* is a diffusional process.

In this paper, we investigate the kinetics and thermodynamics properties of the electron-transfer reactions occurring between the primary and the secondary donors in cells of *Rb. sphaeroides* with intact periplasmic membranes.

Material and Methods

Cells of *Rb. sphaeroides* strains R26 and Ga, and blue-green and a green mutant, grown in the light under anaerobic conditions, in Hutner medium at 30 °C, were harvested after 48 and 24 h, respectively. In most of the experiments, the bacteria were exposed for 1 min to 0.4 mM benzoquinone and then centrifuged at low speed (5 min, $6000 \times g$). The cells were then washed in 10 mM Tris buffer (pH 8) and resuspended in 10 mM Tris buffer (pH 8) with 7% ficoll (w/w) to reduce sedimentation and light-scattering. Different redox states of the electron carriers were obtained by addition of KCN, an inhibitor of the oxidases, at concentrations ranging from 30 μM to 2 mM. The more reducing conditions were realized by addition of 20 mM glucose plus 3 mg/ml glucose oxidase and bubbling of nitrogen in the cell suspension.

Spectrophotometric measurements were performed with an apparatus similar to the one described in Ref. 25 and improved according to Ref. 26. Actinic excitation was provided by xenon flashes (3 μs half-time duration for intense flashes), filtered through a Kodak

Wratten filter 89B and suitable grey filters to decrease the flash energy when required. The use of actinic xenon flashes limits the time resolution to 25 μs.

Absorption spectra of primary and secondary donors

R26 cells, in the presence of 40 μM myxothiazol, were excited by two saturating flashes 1.5 s apart. The light-induced absorption changes linked to the state P^+ were detected 1 ms after the second actinic flash (Fig. 1). This spectrum, which shows in the 535–565 nm region (inset Fig. 1) the characteristic electrochromic effect of reduced acceptors on bacteriopheophytin, does not display any significant contribution of photooxidized cytochromes. The photooxidation of cytochrome $c_1 + c_2$ (denoted here as cytochrome *ct*) is measured as the difference $\Delta A_{551\,nm} - (\Delta A_{535\,nm} + \Delta A_{565\,nm})/2$. This difference cancels when the absorption changes are only associated with P oxidation (see Fig. 1). The concentration of the photooxidized primary donor P was estimated by measuring the difference $\Delta A_{603\,nm} - \frac{2}{3}\,\Delta A_{586\,nm}$. This procedure eliminates a small contribution of cytochrome *ct* in this region.

The extinction coefficients for the cytochromes *c* and P are not known in the case of whole cells, because of possible occurrence of 'sieve effect' [27] which depends mainly upon the absorption of the bacteriochlorophyll antenna.

To estimate the relative contributions of cytochrome c_2 and c_1 in the α-band region, we first confirmed that the oxidized-minus-reduced difference spectra of these two cytochromes were very similar in whole cells and in chromatophores. Absorbance changes linked to the photooxidation of cytochrome c_2 in whole cells were measured 25 μs after a saturating actinic flash. Its absorption peak was found centered at 550.5 nm. The wavelength of the maximum absorption of the α-band of cytochrome c_1 was obtained from measurements of light-induced absorption changes with whole cells of *Rb. capsulatus* mutant MTG4/S4 which lacks soluble cytochrome c_2, a generous gift of Dr. F. Daldal. We found 551.5 nm for its peak wavelength in the α-band (see also Ref. 1). Both absorption maxima of cytochrome c_2 and c_1 are in good agreement with those determined by Meinhardt and Crofts [11] in chromatophores. The ratio $R = (\Delta A_{548\,nm} - \Delta A_{554\,nm}/\Delta A_{551\,nm}$, which is equal to -0.32 ± 0.01 for cytochrome c_2 and $+0.11 \pm 0.01$ for cytochrome c_1, was used as an indicator of the proportion of cytochrome c_2 and cytochrome c_1.

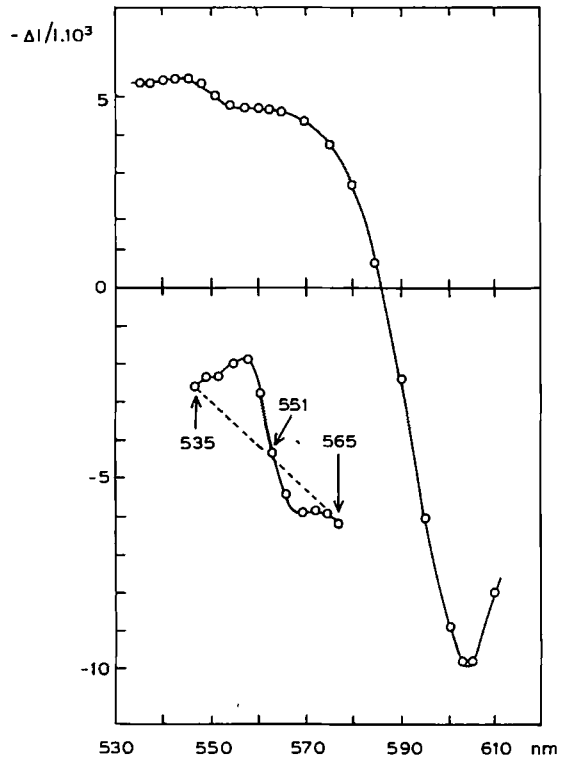


Fig. 1. Difference spectrum of the absorbance changes induced by the second flash of a group of two saturating actinic flashes 1.5 s apart. Detection at 1 ms after the flash. Benzoquinone-treated R26 cells in the presence of 40 μM myxothiazol. Inset: magnification of the 535–565 nm region.

Results and Discussion

Photooxidation of primary and secondary electron donors under different redox potential conditions

The rapid cyclic electron flow, which occurs in photosynthetic bacteria after a single saturating flash, renders difficult a detailed study of the electron transfer at the donor side of the photochemical reaction center. For example, photooxidized cytochrome *ct* is fully rereduced in about 20 ms under reducing conditions (Fig. 2A) [17]. Addition of myxothiazol, an antibiotic inhibitor, was shown to inhibit the electron transfer at the ubiquinol oxidizing site of the cytochrome bc_1 complex in chromatophores [13]. Fig. 2B shows that, also in whole cells, addition of myxothiazol slows down the rereduction of cytochrome *ct* ($t_{1/2} \approx 110$ ms). However, the maximal effect of myxothiazol is obtained for considerably higher concentrations (160 μM) than those used in chromatophores (3 μM) [13].

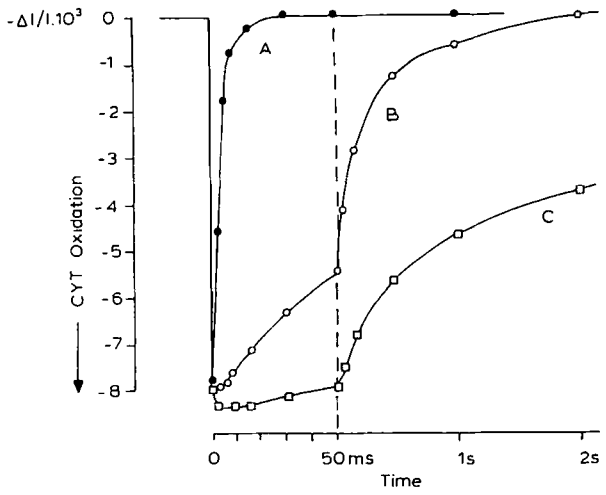


Fig. 2. Flash-induced absorbance changes linked to cytochrome ct in R26 cells under anaerobiosis. A ●, untreated cells; B ○, untreated cells in the presence of 160 μM myxothiazol; C □, benzoquinone-treated cells in the presence of 40 μM myxothiazol. CYT, cytochrome.

In microalgae, benzoquinone treatment is known to make the cell and the chloroplast envelope more permeant to ions, to inhibit the ATPase and the respiratory pathway [28–31]. We observed similar effects on bacterial cells when treated with benzoquinone: first, the decay of the membrane potential as measured by the carotenoid band shift at 505–490 nm in *Rb. sphaeroides* Ga cells is slowed down; the half-times are equal to 80 ms for untreated cells and 350 ms for benzoquinone-treated cells, respectively (data not shown). Second, the respiratory activity measured with a Clark electrode is almost completely inhibited after benzoquinone treatment, but can be restored by addition of exogenous electron donors such as reduced TMPD, showing that benzoquinone treatment affects the reducing pathway of quinones but not the oxidative pathway (cytochrome oxidase). Moreover, benzoquinone treatment renders addition of myxothiazol more effective. Indeed, the maximum inhibitory effect is obtained for lower concentrations (40 μM instead of 160 μM for untreated cells) and the rereduction of cytochrome *ct* is much slower ($t_{1/2} \approx 1.5$ s, compare kinetics B and C, Fig. 2). For these reasons, complete oxidation of primary and secondary donors can be achieved even under weak continuous illumination (approx. 15 photons per center per s); in these conditions the oxidation is limited by the light reaction, since the rate for the electron transfer between primary and secondary donors is much faster than the photochemical rate (Figs. 2 and 7). As a consequence, during the course of illumination, the amount of oxidized P and cytochrome *ct* depends only on the total absorbed energy, i.e., the *It* product, in a broad range of light intensity (from 15 to 100 photons per center per s, data not shown). We checked that during the course of illumination, no appreciable absorption changes could be ascribed to *b*-cytochromes. We can thus exclude any leak of electrons along the cytochromes *b* chain.

In dark-adapted material, the redox potential is controlled by the competition between the rate of reduction of the ubiquinone pool by the substrate and its oxidation rate by the oxidases. In benzoquinone-treated cells, due to the slowing down of the reduction rate, the redox potential is close to 400 mV (as shown by the presence of about 85% reduced P). Modulation of the cytochrome oxidase activity by addition of KCN induces different redox conditions. A full reduction of the cytochromes is obtained under anaerobiosis in the presence of glucose/glucose oxidase.

Fig. 3 (A and B) shows the kinetics of cytochrome *ct* and P oxidation upon continuous illumination after 2 min dark adaptation for various concentrations of KCN, i.e., various initial redox conditions. The following conclusions can be drawn from these experiments (1) The initial slope of P oxidation under anaerobic conditions (Fig. 3B, Curve 1) is close to 0; this implies that all the P's can transfer positive charges to cytochrome *c* (2) The value of *R*, an indicator of the proportion of oxidized cytochrome c_2 and cytochrome c_1 (see Material and Methods), is about constant during the illumination ($-0.05--0.1$). This value corresponds to about equal amounts of photooxidized cytochrome c_2 and c_1 and was found independent of the dark initial redox state. Similar results were obtained with untreated cells. One can thus conclude that the value of the equilibrium constant between cytochrome c_2 and cytochrome c_1 is close to 1, in contradiction with titration data for chromatophores reported in the literature [11]. (3) Under the more reducing conditions, the kinetics of cytochrome *ct* oxidation displays a small lag (Fig. 3, curve 1), which suggests that the cytochromes are in equilibrium with donors of lower potential, most probably the Rieske FeS center. An equilibrium constant between cytochrome *ct* and the FeS center in the range of 2 and 5 accounts satisfactorily for the lag period.

The function [P] = *f*[cytochrome *ct*], computed from the experimental results of Fig. 3 was plotted in Fig. 4.

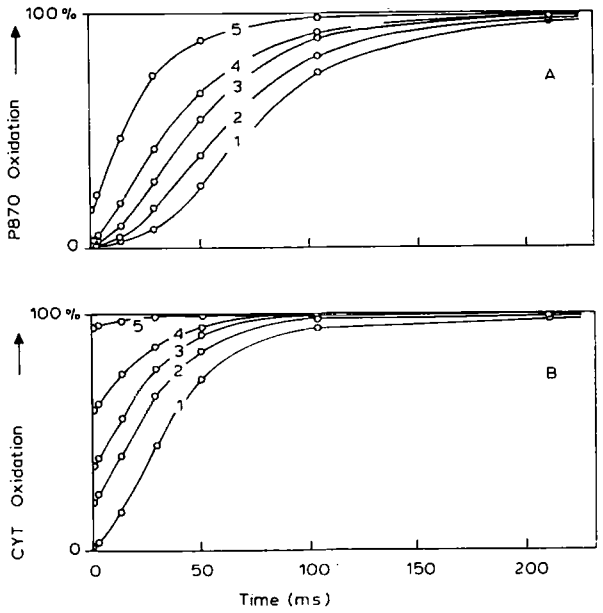


Fig. 3. Photooxidation kinetics of the primary donor P (part A) and cytochrome *ct* (part B) induced by continuous illumination of benzoquinone-treated R26 cells poised at various initial redox potentials. 40 μM myxothiazol. (1) Anaerobiosis obtained by addition of glucose/glucose oxidase +2 mM KCN; (2) 600 μM KCN; (3) 180 μM KCN; (4) 30 μM KCN; (5) No KCN. CYT, cytochrome.

A theoretical curve computed for an equilibrium constant of 70 (solid curve) reasonably fits the experimental points obtained after 2 min dark adaptation (filled symbols) in the presence of different KCN concentrations. This value of 70 for the equilibrium constant corresponds to a difference of 110 mV in the midpoint potentials, in good agreement with the reported values for P and cytochrome c_2 midpoint potentials [6,7,17].

During the course of illumination and whatever the starting redox conditions, the functions [P] = *f*[cytochrome *ct*] (Fig. 4, open symbols) strongly differ from the one measured in dark-adapted conditions. These functions were unchanged for light intensities varying from 15 to 100 photons per center per s, which suggests that quasi-equilibrium is achieved in the donor chain. Nevertheless, the proportion of P^+ during the course of illumination is much larger than what can be predicted from the value of the equilibrium constant. This behavior is inconsistent with a simple model in which all the primary and secondary donors would be in rapid equilibrium. If this were the case, one would expect all the experimental points to lie on the theoretical equilibrium curve (Fig. 4, solid curve) as was the case for dark-adapted material.

In Fig. 5, we compare the functions [P] = *f*[cytochrome *ct*] during cytochrome *ct* and P photooxidation (curve 1) and during their subsequent reduction in the dark (curve 2). Contrary to what was observed during the photooxidation phase, the electron carriers remain close to thermodynamic equilibrium during the reduction phase, as shown by the agreement with the computed curve for $K = 70$.

In Fig. 6, the amounts of photooxidized cytochrome *ct* and P were plotted (closed symbols) for benzoquinone-treated cells submitted to a series of six short saturating flashes 80 ms apart. Even under the more reducing conditions (curve 1), the first flash induces the formation of an appreciable amount of P^+, stable in the millisecond range (about 12%) although less than 50% of cytochrome *ct* was photooxidized by this flash. This fraction is somewhat variable from one R26 culture to another (10–20%); in the case of Ga cells, this amount can reach 40%. Similar observations were reported in the literature for both whole cells and chromatophores [1,4].

Models

From the preceding experiments, we conclude that the different electron donors are not in thermodynamic equilibrium during the photooxidation phase. Models in which reaction centers, cytochrome c_2 and cytochrome bc_1 complexes are associated in 'supercomplexes' may account for our experimental data. On a short time scale (less than 1 s), equilibrium would be achieved independently within each supercomplex, whereas redox equilibration between supercomplexes, mediated by diffusing molecules, would only occur on a longer time scale.

The formation of stable P^+ following a single saturating flash (Fig. 6) can be interpreted by assuming that a fraction of the reaction centers is included in supercomplexes formed by the association of two reaction centers ('duplex') with one cytochrome c_2. In these supercomplexes denoted here as $P\text{–}P/c_2$, only half of the photooxidized primary donor would be rereduced by cytochrome c_2, leading to the state $P\text{–}P^+/c_2^+$. On the other hand, after illumination by a weak flash, the probability for a double excitation within the same duplex is negligible; therefore, any P^+ will be rereduced by cytochrome c_2, consistently with the lag observed for P oxidation (Fig. 3A, curve 1). We assume that the remaining fraction of reaction centers takes part in

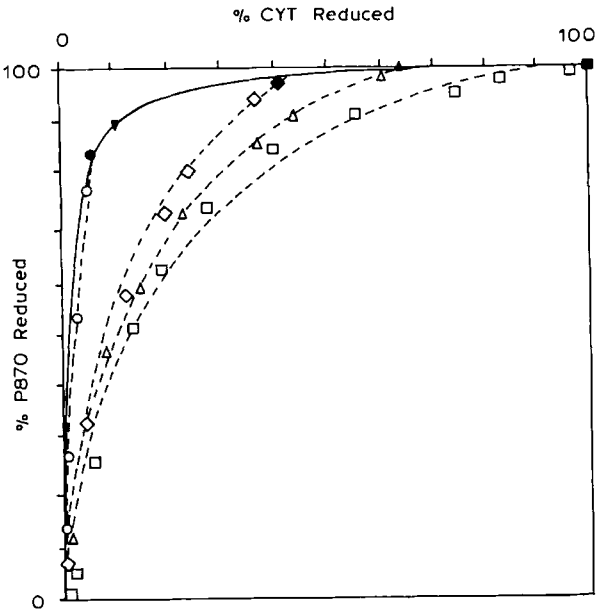


Fig. 4. Percentage of reduced cytochrome *ct* as a function of reduced P using data from the experiments of Fig. 3. Filled symbols: after 2 min dark adaptation; open symbols; during the course of continuous illumination. ■, □, anaerobiosis, glucose/glucose oxidase + 2 mM KCN; ▲, △, 180 μM KCN; ◆, ◇, 30 μM KCN; ▼, 10 μM KCN; ●, ○, no KCN. Solid line: theoretical curve drawn for an equilibrium constant of 70 between P and cytochrome *ct*. Dashed lines: theoretical curves computed for the photooxidation (see text). CYT, cyto chrome.

supercomplexes which include two reaction centers, one cytochrome c_2 and one bc_1 complex (P–P/c_2/c_1 FeS). This hypothesis is in line with several suggestions that (1) the concentration of the reaction centers is about two times larger than that of cytochrome bc_1 complexes [1–4]; (2) close associations between reaction centers, cytochrome c_2 and bc_1 complexes can exist in chromatophores membranes [21,22].

A theoretical analysis of the charge distribution within supercomplexes during the photooxidation process induced by continuous illumination or flash excitation is extensively described in the accompanying article [32]. The parameters used in this theoretical calculation are the equilibrium constants between the different donors (as obtained in dark-adapted conditions), the ratio between the two types of supercomplexes (P–P/c_2) and (P–P/c_2/c_1 FeS), and the 'miss' coefficient α. Upon illumination by a saturating flash. $1-\alpha$ represents the probability of stabilizing one positive equivalent per reaction center. The value of α was measured by analyzing the damping of the period 2 oscillations in the formation of the secondary semiquinone, detected at 450 nm in the presence of 10 mM reduced TMPD as an electron donor. In benzoquinone-treated cells, and taking into account some variability from batch to batch, we found $\alpha = 0.12 \pm 0.02$ (data not shown); for the sake of simplicity, we consider α as a constant whatever the flash number. As already discussed, the following values for the different equilibrium constants were determined: $K_1 = 70$ (P/cytochrome c_2); $K_2 = 1$ (cytochromec_2/cytochrome c_1); $K_3 = 2.5$ (cytochrome c_1/FeS). The equilibrium constant between P and cytochrome c_2 is in reasonable agreement with the midpoint potential values reported in the literature [6,7,17]. The main discrepancies concern the midpoint potentials of cytochrome c_1 and FeS, which here appear approx. 75 mV and approx. 35 mV, respectively, more positive than expected from titration data [7,11].

The proportion of reaction centers included in P–P/c_2 supercomplexes was estimated from the relative amount (12%) of stable P^+ after the first flash in the more reducing conditions (Fig. 6). Taking into account the miss coefficient and the fact that, in such complexes, half of the P^+'s is rapidly reduced by cytochrome c_2, the proportion of P–P/c_2 supercomplexes was found equal to 0.27.

Using this set of parameters, the theoretical functions [P] = f[cytochrome *ct*] were computed during the course of a continuous illumination for various initial redox conditions. The fit with experimental data is excellent (Fig. 4), whatever the starting redox conditions.

The amount of photooxidized cytochrome *ct* and P following a series of saturating flashes was computed according to the same mathematical model (Fig. 6, open symbols). Although the general behavior of the evolution of the flash-induced oxidation of P and cytochrome *ct* is correctly described, minor discrepancies can be noticed (1) The theoretical calculation does not take into account the rereduction of P and cytochromes which occurs between flashes, especially under the more reducing conditions. This explains why the overall yield of photooxidation of primary and secondary donors is significantly higher in the theoretical than in the experimental case. 2) The computed value for the amount of oxidized cytochrome *ct* following the first flash is 5% to 10% higher than the experimental one. A better fit should be obtained by assuming that the value of the equilibrium constant between P and cytochrome *ct* is lower after a flash than in dark-adapted conditions.

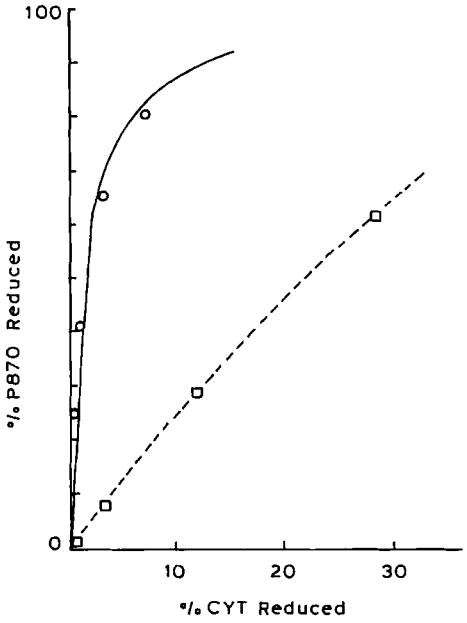


Fig. 5. Percentage of reduced P as a function of reduced cytochrome *ct*. Benzoquinone-treated R26 cells under anaerobiosis. □, at the end of the continuous illumination; ○, during re-reduction in the dark. Solid line: equilibrium curve computed for an equilibrium constant of 70 between P and cytochrome *ct*. Note the twofold extended scale for cytochrome *ct* compared with that of P. CYT, cytochrome.

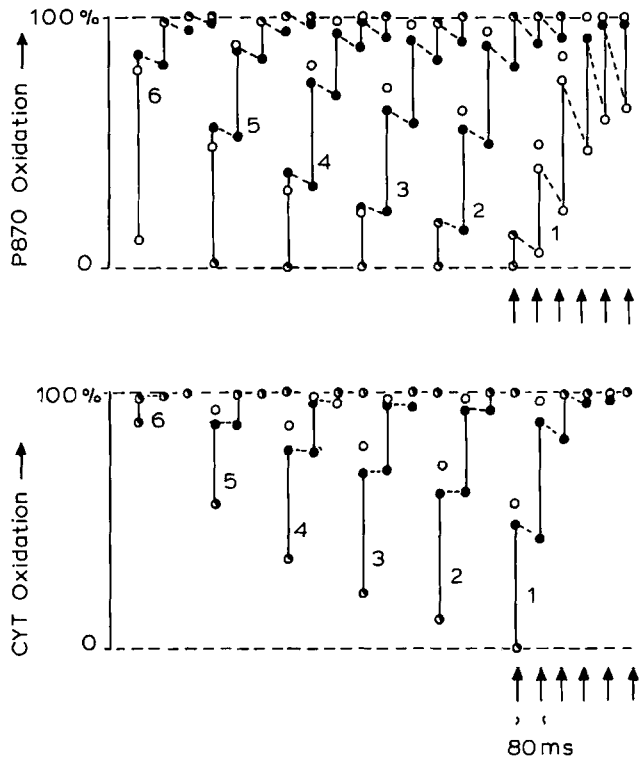


Fig. 6. Absorption change linked to the photooxidation of P (upper part) and cytochrome *ct* (lower part) detected at 1 ms and 80 ms after each flash of a series of six saturating actinic flashes (80 ms apart), at various initial redox potentials. Same benzoquinone-treated R26 cells as in the experiments of Fig. 3. 40 μM myxothiazol. Filled symbols: experimental data; open symbols: values computed from the model developed in the text. (1) anaerobiosis; (2) 2 mM KCN; (3) 600 μM KCN; (4) 180 μM KCN; (5) 30 μM KCN; (6) no KCN. The absorption changes corresponding to a full oxidation of P and cytochrome *ct* starting from anaerobic conditions are equal to $1.68 \cdot 10^{-2}$ and $2.57 \cdot 10^{-2}\ \Delta I/I$, respectively. CYT, cytochrome.

This may actually be expected from an effect of the transmembrane potential present after the flash. Taking into account that about 30% of the electrogenicity of the charge separation [17] is associated with electron transfer from cytochrome c_2 to P and that a membrane potential of about 50–100 mV [33] is developed after a flash, the equilibrium constant between these two carriers should be decreased of a factor 2–3.

It is noteworthy that in the theoretical analysis presented here, the parameters were experimentally determined rather than adjusted a posteriori in order to get the best possible fit.

According to our model and for the batch used in experiments Figs. 2, 3 and 6, the ratio *ct*/P of the number of cytochrome *ct* to the number of primary donors P is 0.87. The ratio of the maximal absorption changes associated with the photooxidation of cytochrome *ct* and P measured after a flash sequence given under reducing conditions (see legend Fig. 6) is equal to 1.54. One may then compute that the ratio of the extinction coefficients ϵ_{ct}/ϵ_P is equal to 1.77. This ratio differs from that evaluated in Refs. 8 and 34 for chromatophores ($\epsilon_{ct}/\epsilon_P \approx 1.15$) but agrees with the evaluation by Prince et al. in the case of living cells [1].

Measuring the singlet–singlet annihilation after an intense laser flash, Vos and coworkers [35,36] conclude that 3–4 reaction centers were in close physical interactions. On the basis of the analysis developed by Lavergne et al. [32], we computed the theoretical functions [P] = *f*[cytochrome *ct*] for clusters including either two or four reaction centers. The agreement with the experimental data is definitely better when considering dimers rather than tetramers, specially when a fraction of the secondary donors is in the oxidized state at the onset of the illumination. Therefore, it is very unlikely that one

cytochrome c_2 could rapidly react with more than two reaction centers.

Kinetics of electron transfer upon flash excitation

Fig. 7A shows the time-course of photooxidation of the cytochromes and re-reduction of P^+ following a single saturating flash. The experiment was performed in untreated cells (similar results were obtained with benzoquinone-treated cells). The photooxidation of cytochrome *ct* presents two phases which are correlated with the rereduction of P^+. The first phase is completed in less than 25 μs, while the half-time for the second phase is about 130 μs, in agreement with previous results [8,10,37]. As already noticed, part of photooxidized P (here, about 20%) is not rapidly reduced. During the fast phase of P^+ reduction, oxidation of the sole cytochrome c_2 occurs, while during the subsequent phase, electrons are transferred from cytochrome c_1 to cytochrome c_2, as shown by the variations of the ratio R (Fig. 7, lower trace). The value of R measured at times longer than 1 ms indicates that there is about the same amount of oxidized cytochromes c_1 and c_2. Due to the duration of our xenon flashes, the fast reduction phase of P^+ could not be resolved. Nevertheless, its amplitude could be estimated by measuring the difference between the absorption change induced by a strong continuous illumination, which fully oxidized P and the absorption change measured 25 μs after a saturating flash. Assuming that during the fast phase, the amount of reduced P is equal to the amount of oxidized cytochrome c_2, one may compute a ratio $\epsilon_{c_2}/\epsilon_P$ equal to 1.6–1.8, depending upon the value of the miss coefficient on the first flash. This ratio agrees with the one previously computed on the basis of our structural model (1.77).

Fig. 7B shows the time-course of photooxidation of cytochrome *ct* and rereduction of P^+ following a single weak actinic flash, which hits about 5% of the centers. In these conditions, all photooxidized P is reduced in less than 500 μs. This result agrees with the experiments

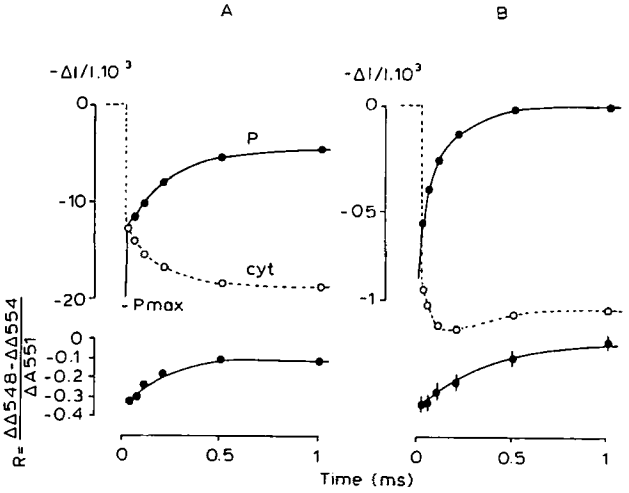


Fig. 7. Kinetics of flash-induced redox changes of P (●) and cytochrome *ct* (○). 160 μM myxothiazol; 500 μM KCN. Lower traces: the ratio $R = (\Delta A_{548\,nm} - \Delta A_{554\,nm})/\Delta A_{551\,nm}$ gives an estimate of the respective contribution of cytochrome c_2 and cytochrome c_1 (see Material and Methods section). (A) Saturating actinic flash; (B) weak actinic flash (notice the different vertical scales). cyt, cytochrome.

using continuous light, where the value of the initial slope for P photooxidation is close to zero (Fig. 3A, curve 1). As to the slower reduction phase ($t_{1/2} \approx 60$ μs) of P^+, a two-fold acceleration was found compared with saturating excitation. Moreover, there is no longer any correlation between the reduction kinetics of P^+ and the kinetics of electron transfer between cytochrome c_2 and c_1, as measured by the variation of the ratio R.

The preceding observations can be interpreted according to the supercomplexes hypothesis. Within a P–P/c_2/c_1 FeS supercomplex, cytochrome c_2 has, at a given time, an efficient interaction for electron transfer only with one of its three partners, since the same proteic domaine of cytochrome c_2 is involved in the redox reactions [38]. Following the charge separation induced by a saturating flash, most of the supercomplexes have their two P's in the oxidized state (Scheme I).

$$\begin{matrix} Pc_2 \\ P \end{matrix} c_1FeS \xrightarrow{h\nu} \begin{matrix} P^+c_2 \\ P^+ \end{matrix} c_1FeS \xrightarrow{3\,\mu s} \begin{matrix} Pc_2^+ \\ P^+ \end{matrix} c_1FeS \longrightarrow \begin{matrix} Pc_2 \\ P^+ \end{matrix} c_1^+FeS \longrightarrow \begin{matrix} P \\ P^+c_2 \end{matrix} c_1^+FeS \longrightarrow \begin{matrix} P \\ Pc_2^+ \end{matrix} c_1^+FeS$$

$$\text{overall } t_{1/2} = 130\ \mu s$$

Scheme I

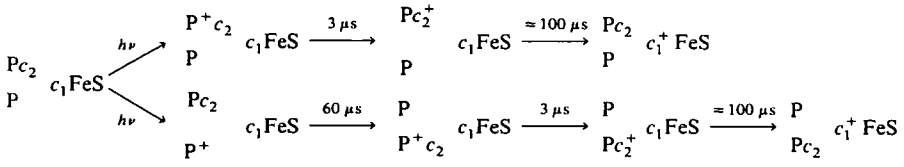
In less than 25 μs, the photooxidized P interacting with cytochrome c_2 will be reduced. The half-time for this fast phase was estimated to about 3 μs [8,10,37]. For the P–P/c_2 centers, our model predicts that 50% of the P's are associated with a cytochrome c_2 molecule. For the P–P/c_2/c_1 FeS centers, the fraction of P associated with cytochrome c_2 is expected to be lower than 50%, depending upon the relative affinity of cytochrome c_2 for the cytochrome bc_1 complex and reaction center. This agrees with the fact that less than 50% of P^+ is reduced in a time shorter than 25 μs. The reduction of the remaining fraction of P^+ would be possible only after (1) rereduction of cytochrome c_2 by cytochrome c_1 and (2) reassociation of this reduced cytochrome c_2 with the second P^+ present in the same 'duplex'. The concomitant reduction of P^+ and oxidation of cytochrome c_1 shows that the reduction rate of P^+ is actually mainly limited by the electron-transfer reactions between cytochrome c_2 and cytochrome c_1.

Following a weak actinic flash, the probability to photooxidize two P's within the same supercomplex is very low (Scheme II).

For the fraction of P^+ in tight association with cytochrome c_2, the reduction occurs in less than 25 μs. The reduction of another fraction of P^+ ($t_{1/2} \approx 60$ μs) is limited by the time for cytochrome c_2 to switch from one reaction center to the other within the same 'duplex'. This switching time is independent of the electron-transfer rate from cytochrome c_1 to cytochrome c_2. For the P–P/c_2/c_1 FeS centers, the 60 μs phase of cytochrome c_2 oxidation is followed by a reduction phase associated with the electron transfer from cytochrome c_1 to FeS. In these supercomplexes, a major fraction of the positive charges is finally stabilized in the FeS center. This accounts for the small reduction of cytochrome *ct* observed at times longer than 200 μs. A third component in the reduction kinetics of P^+ could be associated with the switching of cytochrome c_2 from cytochrome bc_1 complexes to reaction centers. Since the major fraction of cytochrome c_2 is associated with a reaction center, this third phase would be of too small amplitude to be detected.

The analysis presented here requires that the diffusion of one molecule of cytochrome c_2 is restricted to a domain including two reaction centers and one cytochrome bc_1 complex. The effect of the viscosity of the medium on the rate of oxidation of cytochromes in whole cells, as reported by Van der Wal et al. [39], suggests that the rate of diffusion of cytochrome c_2 within a supercomplex is a limiting factor. The most likely hypothesis to account for this restricted diffusion of cytochrome c_2 is that the three membrane-bound partners are associated in a well-defined structure. In vitro experiments have shown that cytochrome c_2 specifically binds to isolated reaction centers with an affinity which depends upon the ionic strength of the medium [10,17,38]. Interactions between two reaction centers or between reaction center and cytochrome bc_1 complexes could occur either directly or via the core antenna complex which surrounds the reaction center. A scheme of the two types of supercomplexes, which intends by no means to be an accurate structural representation, is shown in Fig. 8. In these models, cytochrome c_2 can occupy two or three sites, depending upon the type of supercomplexes. It is necessary to assume that the binding of only one molecule of cytochrome c_2 on one of the three sites available in the supercomplex prevents the association of other cytochrome c_2 molecules.

It is noteworthy that the proportion of reaction centers included in P–P/c_2 and P–P/c_2/c_1 FeS supercomplexes widely varies depending upon the conditions of culture and the mutant strain. This suggests that the formation and the dissociation of supercomplexes are controlled by regulatory processes. Preliminary results obtained with whole cells of *Rb. sphaeroides* Ga strain show that a fraction which can reach 40% of the reaction centers is included in P–P/c_2 centers. In this case, cytochrome c_2 could slowly escape from these supercomplexes and reacts with cytochrome bc_1 complexes



Scheme II

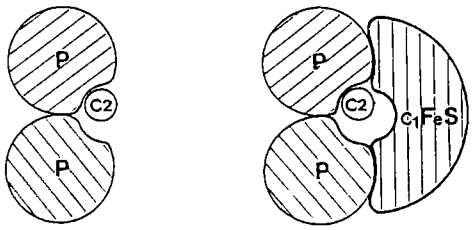


Fig. 8. Schematic representation of P–P/c_2 and P–P/c_2/c_1FeS supercomplexes.

located in another area of the membrane by a diffusion-controlled process. For some bacterial cultures, we observed, in contrast with the experiment of Fig. 7, that a weak flash induces the formation of a significant amount of stable P^+ (up to 10%), which shows that a fraction of the reaction centers is disconnected from cytochrome c_2. This suggests that in such cases, the available amount of cytochrome c_2 is lower than the amount of 'duplex' P–P.

It is worth mentioning here that stable supercomplexes between cytochrome bc_1 and cytochrome oxidase were isolated from *Paracoccus denitrificans* [40] and from the thermophilic bacteria PS3 [24]. Formation of supercomplexes between electron carriers might be a general feature in energy conserving membranes.

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