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KINETIC ANALYSIS OF BACTERIAL REACTION CENTERS RECONSTITUTED IN LIPID BILAYERS

H.-J. APELL [a], M. SNOZZI [b,*] and R. BACHOFEN [b]

[a] *Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz (F.R.G.) and* [b] *Institut für Pflanzenbiologie, Universität Zürich, CH-8008 Zürich (Switzerland)*

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(1) Reaction center-lipid complexes were extracted into octane solutions. Different methods for generating an assymetric membrane distribution of reaction centers are discussed, which allow the measurement of electrical signals upon illumination. (2) The dichroism of the chromophoric groups in the reaction centers was investigated in planar lipid bilayers and the angle β between each transition moment and the normal to the membrane could be determined to be β(757 nm) = 29.5 ± 1.2, β(801 nm) = 34 ± 1.0 and β(860 nm) = 41.3 ± 0.9°. (3) The kinetics of the reaction centers from *Rhodopseudomonas sphaeroides* were analysed by electrical measurements and the relevant rate constants could be determined. In addition, the interaction between reaction centers and the intramembrane, ubiquinone-containing pool was investigated and described in a kinetic model. (4) The interaction between the electron-donating ferrocytochromes exhibited two distinguishable sources, a fast accessible, membrane-bound pool, which is limited by diffusion, and a pool consisting of an aqueous solution of ferrocytochrome *c*, which is accessible with a slower rate constant.

Introduction

Photosynthetic bacteria are able to produce metabolic energy from absorbed light. The light-induced activity causes an electric charge separation and a redox potential across the plasma membrane of the bacteria generated by reaction center proteins. The photosynthetic apparatus of the bacteria is mainly located in tubular invaginations of the membrane, the chromatophores (for a review see 1). The reaction center has an approximate molecular weight of 90000 and contains beside three protein subunits several pigments: one bacteriochlorophyll dimer, two bacteriochlorophylls and two bacteriopheophytins. The reaction centers have also two binding sites for ubiquinone which are close to the cytoplasmic surface of the membrane. At the periplasmic side the reaction centers have binding sites for two cytochromes c_2, so that the proteins span the entire membrane [2].

The absorption of a photon leads to the transfer of an electron initially from the bacteriochlorophyll dimer via one of the pheophytins to the primary acceptor, the ubiquinone Q_I and subsequently to the secondary ubiquinone Q_{II}. In the native membrane the oxidized bacteriochlorophyll dimer is reduced by a cytochrome c_2 bound to the reaction center. Q_{II} acts as a two-electron gate, its oxidized and completely reduced forms being in equilibrium with the quinone pool in the chromatophores [3]. From the secondary ubiquinone

* Present address: Department of Physiology and Biophysics, University of Illinois, Urbana, IL, U.S.A.

Abbreviation: Mops, 3-(*N*-morpholino)propanesulfonic acid.

the electron returns to the oxidized cytochrome c_2 through a pathway involving the cytochrome b-c_1 complex [4]. The cyclic electron flow is coupled to a vectorial transport of protons from the cytoplasm to the periplasm across the membrane.

Investigations of the kinetic properties of the reaction center were effected with whole chromatophores and detergent-solubulized reaction centers using different spectroscopic methods. Electron transfer from the donating bacteriochlorophyll dimer to the primary acceptor Q_I requires less than 150 ps [5]. The direction is perpendicular to the membrane plane and, in this manner, generates a potential [6,7]. Subsequent electron transfer to the secondary acceptor Q_{II} is not electrogenic and occurs in less than 1 ms [8]. The donating cytochrome c_2 provides an electron to the oxidized reaction center within micro- to milliseconds [9]. Investigations of the electrical properties of reaction centers were carried out with chromatophores and isolated reaction centers in different systems. Drachev et al. [19,11] adsorbed chromatophores and vesicles with incorporated reaction centers onto thick membranes and observed photovoltages and photocurrents. Progress was made by reconstitution of isolated reaction centers in lipid bilayer membranes [12–14] and with monolayers formed from isolated reaction centers deposited between metal electrode films [15,16].

The emphasis of this paper is to evaluate quantitatively the reaction center kinetics under multiple-turnover conditions. This was done on the basis of the electric responses of the membrane-reconstituted reaction centers and its adjacent electron donors and acceptors.

Materials and Methods

Cultivation of bacteria and isolation of reaction centers

Cells of the carotenoidless mutant *Rhodopseudomonas sphaeroides* R26 were grown in 10-l bottles at 30°C using the medium of Ormerod et al. [17] supplemented with *p*-aminobenzoic acid, thiamine, nicotinic acid and 0.8% yeast extract. The growing conditions, harvesting and the preparation of the chromatophores were performed as described by Snozzi and Bachofen [18]. The reaction centers were isolated and purified using procedures described previously [19,20]. The purity was checked by SDS-polyacrylamide gel electrophoresis and by spectroscopic analysis and corresponded with published data [21].

Preparation of the reaction center / lipid octane solution

Similar procedures were used to those of Schönfeld et al. [12,22] and Packham et al. [14]. To prevent oxidation of the isolated reaction centers all steps were performed in darkness or in dim green light. The exclusion of oxygen by flushing the atmosphere above the preparation with nitrogen or argon gas did not produce any detectable difference in the absorption spectra of the final solution. The primary lipid used was L-α-phosphatidylcholine derived from soybeans (Sigma) and used without further purification. For preparations with different lipid compositions, mixtures of phosphatidylethanolamine were used in various molar ratios. Maximum yield, in terms of optical absorption at 800 nm and the ratio of absorption, $A_{800\ nm}/A_{757\ nm}$, was obtained by the following procedure. 10 mg L-α-phosphatidylcholine were sonicated in 1 ml of 5 mM Mops buffer, pH 7, for 30 min at 40°C in a bath sonicator (Bransonic 12-60 W). The vesicles were then cooled on ice and an aliquot of reaction centers was added. A typical concentration of reaction centers was 0.52 mg protein/10 mg lipid. Because of a strong dependence of the protein concentration on yield (Fig. 1) for each newly isolated batch of reaction centers, the maximum yield was determined. The vesicle/reaction center solution was kept overnight on ice. After sonication of the reaction center/vesicle solution for 8–10 min, 2 ml *n*-octane and 1 M $CaCl_2$, to produce a final concentration of 100 mM, were added and vortex mixed for 4 min. The milky emulsion was centrifuged at 5000 rpm for 5 min to a phase separation consisting of a compact sediment and the clear octane supernatant fraction. This supernatant fraction was used to repeat the extraction from a second sample of reaction center/vesicles. This repetitive step increased the absorption at 800 nm by a factor of 2.3–2.5. The total yield of transferred reaction centers was typically 35–40%, equivalent to 3.6 nmol reaction center. The quality of the reaction center/lipid octane solution was checked by the absorption

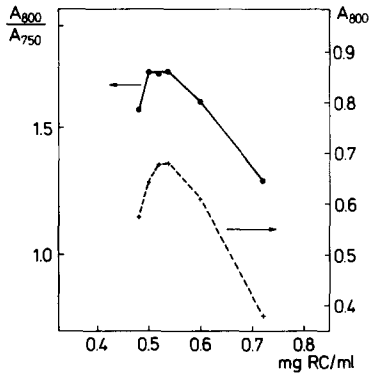


Fig. 1. Efficiency of extraction of reaction center-lipid complexes into *n*-octane as a function of protein/lipid ratio. (+ — — — +). Absorption of the solution at 800 nm; (●———●) ratio of absorptions, $A_{800\ nm}/A_{757\ nm}$. The curves are data connecting lines.

ratio $A_{800\ nm}/A_{757\ nm}$. This is an indicator of the quality of the solution as the content of intact reaction centers at 800 nm and the amount of free bacteriochlorophyll, absorbing at 760 nm, can be determined. Typical values were (a) for intact, detergent-solubilized reaction centers 2.2, (b) for reaction center-loaded vesicles 2.1–2.2, and (c) for octane reaction center-lipid complexes 1.7–1.8.

Membrane experiments

The setup for membrane experiments was mounted on an optical bench, placed on a vibration-insulating table. The light source was a 250 W halogen lamp powered by a stabilized d.c. power supply (HP, Harrison, G274A). Using lenses and apertures the light was focused onto the membrane. 5 cm of water were used to protect the equipment against heat. Depending on the experiment, narrow-band metal interference filters (Balzers, Lichtenstein) and/or a polarization filter could be inserted into the light path. An electronical shutter (Prontor Electronic-m) which was computer controlled for synchronization was used to determine the time of illumination. The membrane cell consisted of black Teflon in which the membrane plane was inclined at 45° to the incident light beam. The entrance window was perpendicular to the light. A second window allowed monitoring of the membrane under reflecting light. Maximum light intensity with white light was 2.2 W/cm^2, when the light was focused to a spot of 1.5 mm diameter. The intensity was measured with a radiometer (Yellow Springs Instruments, OH, Model 65A). Black Teflon was used to reduce possible effects of reflected light. The electrolyte could be stirred in both compartments. The electrolyte-containing cuvette could be thermostatically controlled between 2 and 40°C, but if not explicitly mentioned, the temperature was 23°C. The electrodes used were commercially available silver/silver chloride electrodes (IVM Systems, Healdsbury, CA) which were shielded against light by a black plastic tube. The electrodes were connected via shielded cables with a current amplifier (Keithley Model 427) employing a gain of 10^8–10^9 and a rise time of 0.03–1 ms. The output signal was monitored on a storage oscilloscope and, for further processing, connected to the analog input of an analog-digital module of an MINC 11/23 computer which was used for data collection, averaging and storage. The maximum data sampling rate was 20 kHz. A KIM-1 microcomputer was used for synchronisation of the shutter and oscilloscope trigger with the MINC computer.

The membrane cuvette was mounted in a Faraday cage to ensure that the total electrical noise was less than 20 pA when used with a membrane of 1.2 mm diameter at zero voltage and 1 ms time resolution. If, during the experiments, the processes were reversible, data averaging was also used to increase the signal-to-noise ratio. Optically black membranes with membrane areas between 0.008 and 0.03 cm^2 were formed as described elsewhere [23]. The experiments were performed in a buffer of 10 mM NaCl and 10 mM Tris-HCl, pH 8, with membranes from the octane reaction center-lipid complexes, which were concentrated to 2–5% lipid (w/v) by evaporating the octane with a nitrogen stream. As expected these membranes did not show any light-induced current signal, since the reaction centers are symmetrically distribution in the membrane. These experiments also demonstrates that the light shielding of the electrode was sufficient.

To obtain an asymmetric system two methods were used: (1) Addition of potassium ferricyanide to one side of the membrane to a final concentration of 1.23 mM was used to oxidize that part of the population of reaction centers which are ex-

posed to the periplasmic end of the protein: or (2) addition of ferrocytochrome *c* (Sigma, horse heart, type IV) to the other side of the membrane was used to enable multiple electron turnovers of one part of the reaction center population. The ferrocytochrome *c* stock solution contained traces of sodium dithionite and was kept under nitrogen to keep the cytochrome in its reduced from. As shown in Fig. 2 the effects of both addition were additive.

As described earlier [13,22], the procedure of transferring reaction center-lipid complexes into an octane (hexane) phase leads to a dislodging of ubiquinones from the reaction center. The high dilution of the electron acceptors in the bulk hinders a recombination so that, as a consequence, only a small photoresponse can be observed in membrane experiments. To ensure almost complete restoration of the electron acceptor, Q_{10} (Sigma) was added into the reaction center-lipid octane extract up to a concentration of 2 mM, equivalent to a 1000-fold excess. To facilitate the recombination of the ubiquinones with the reaction centers the solution was sonified in a bath for 10–20 s. For further consideration, it has to be taken into account that the excess Q_{10} formed in addition an acceptor pool which could exchange with the secondary acceptor Q_{II} of the reaction

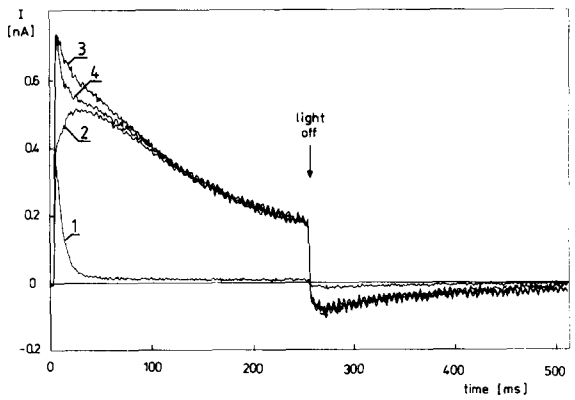


Fig. 2. Photocurrent of a reaction center-containing lipid bilayer membrane: 35 mW/cm^2, $\lambda = 850$ nm, illumination time 250 ms. The experimental data are the average of 10 illuminations. The dark time between two illuminations was 15 s. The asymmetric distribition of reaction centers was generated by (1) 1.25 mM potassium ferricyanide on side I; (2) 12.5 mM ferrocytochrome *c* on side II; (3) 1.25 mM potassium ferricyanide on side I and 12.5 mM ferrocytochrome on side II. Curve 4 is the computed sum of curves 1 and 2.

center. For a few experiments a water-soluble ubiquinone Q_0, an analog without an isoprenoid side chain, was used. This analog was a gift from the Hoffmann-La Roche Co., Basel.

The amplitude of the photocurrent exhibited a time dependence during aging of the membrane. Subsequent to the application of ferrocytochrome *c* or potassium ferricyanide, the amplitude grew to its maximum value within 2–5 min, stayed constant for approx. 10 min and then decreased at a rate of 10% per 25–30 min. Therefore, in certain time intervals, normalization measurements were taken for correction. The time constants of the observed photoprocesses, however, stayed unchanged. Although the electrolyte was always degassed and flushed with argon, the decrease in the signal amplitude could not be reduced significantly. One reason could be the presence of oxygen which was dissolved in the membrane-forming solution, however, performing the complete extraction procedure under a nitrogen atmosphere failed to prevent the decrease in amplitude. A second reason for this effect may be a migration of the reaction centers into the bulky torus of the membrane, where random orientation of the reaction centers would cancel any net component that contributes to the current. To check the contribution of the torus to the photocurrent a small shield was placed directly in front of the black membrane at a distance of less than 1 mm. By turning the black plastic sheet the size of the shaded area was changed. When the bilayer was protected against light but not the torus only 3% of the current amplitude remained. When the torus was also protected, the current was reduced to 1%. Thus, these portions of current could be attributed to reflections within the system.

Results

Intensity dependence of photosignals

To check that the observed photosignals were generated by the reaction center, the action spectra of reaction center-containing membranes were recorded. They were found to be in qualitative agreement with the absorption spectrum and previously published data [13,22]. Both the initial peak amplitude of the photocurrent, $I_{ph,o}$, observed after switching on the illumination, and the

amplitude of the backward-directed dark current after the shutter was closed $I_{b,o}$, were determined as a function of light intensity. The light intensity was varied with a series of calibrated grey filters (Balzers, Lichtenstein). In each case, the peak currents showed saturating behavior that could be described by the equation:

$$I = I^s \frac{J}{J + J_{1/2}} \tag{1}$$

where I^s is the saturation current and $J_{1/2}$ the half-saturation intensity. Typical values for $\lambda = 850$ nm were $J_{1/2} = 36$ mW/cm^2 and $I^s_{ph,o} = 54$ nA/cm^2. The ratio of saturating amplitudes was calculated to be $I^s_{ph,o}/I^s_{b,o} = 39$. The results for infrared and white light did not differ significantly; $J_{1/2}$ was determined to be 255 mW/cm^2 and $I^s_{ph,o} = 150$ nA/cm^2.

The time analysis of the photocurrent required two time constants were the contribution of the second time constant to the total signal was always less than 10%. The main process had an experimentally determined time constant, τ_{ex}, which was defined as the time interval between peak current and the time until the photocurrent decreases to $1/e$ of the peak value. This time constant was strongly dependent on light intensity, J, whereas the dark (back) current time constant was insensitive to the variation of J, as shown in Fig. 3. The peak current $I_{ph,o}$ and time constant τ_{ex} exhibit a strikingly similar functional dependence on light intensity.

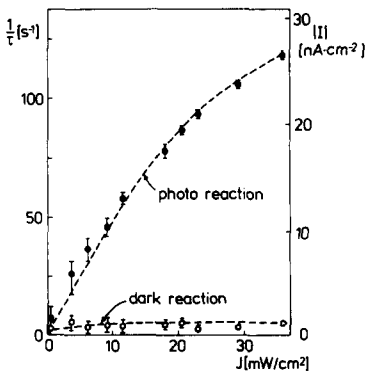


Fig. 3. Time constant τ_{ex} for light and dark current as a function of the light intensity. Light source: 250 W and narrow-band interference filter, $\lambda = 850$ nm, corresponding to 36 mW/cm^2 on the membrane. The points indicate the mean values and bars show the range of single measurements. The curve is the corresponding peak photocurrent $I_{ph,o}$.

Dichroism experiments

Steinemann et al. [24] measured the orientation of the porphyrin ring in artificial chlorophyll-containing membranes. Their technique and theory were applied successfully to bacteriorhodopsin [25] and can be used for reaction centers in the same way. To distinguish between the bacteriochlorophyll, single and special pair, and bacteriopheophytin, narrow-band interference filters were used.

Steinemann et al. [24] used the fact that the absorption cross-section of linearly polarized light is proportional to cos 2ψ, where ψ is the angle between the electrical vector of the exciting light and the transition moment of the chromophore. In the absence of saturation effects, i.e., at sufficiently low light intensity, the photocurrent may be assumed to be proportional to the absorption cross-section. From this assumption the following equations were derived [24]:

$$I(\theta) = I^+ (A + B\cos 2\theta) \tag{2}$$

$$A = \tfrac{1}{2}\sin^2\alpha \sin^2\beta + \tfrac{1}{4}\cos^2\beta (1 + \cos^2\alpha) \tag{3}$$

$$B = \tfrac{1}{4}\sin^2\alpha (3\sin^2\beta - 1) \tag{4}$$

where θ is the angle between the electrical vector of the light and the plane of incidence, α the angle between the normal to the membrane and the light beam, β the angle between the transition moment and the plane of the mebrane, and I^+ a constant proportionality factor.

The dichroic ratio D may be calculated from Eqns. 2–4:

$$D = \frac{I(0)}{I(90)} = 1 + \sin^2\alpha (2\tan^2\beta - 1) \tag{5}$$

With a given construction of the membrane cell the angle between membrane and incident light beam was $\alpha = 45°$. Thus, Eqns. 2–4 can be used to express $I(\theta)/I(45)$ as a linear function of cos 2θ:

$$\frac{I(\theta)}{I(45)} = 1 + \frac{3\sin^2\beta - 1}{3 - \sin^2\beta} \cos 2\theta \tag{6}$$

Depending on the chosen filter the light intensity at the membrane was determined as 15–20 mW/cm^2. In each case, however, the intensity was far below saturation. The experiment was carried out by illuminating the membrane for 1/4 s and evaluating peak current (I) and total charge moved during the light period. The difference between both values, with respect to the ratio of interest $I(\theta)/I(45)$ and $q(\theta)/q(45)$, was insignificant so that finally only the current ratio was used for presentation. It was also necessary to correct the data for the residual polarization of the halogen lamp, which was in the range of 5%. During the experiment, care was taken to ensure that the membrane was kept planar to prevent a reduction of the polarization effects which were observed with a bulging membrane.

In Fig. 4A the experimental values of $I(\theta)/I(45)$ have been plotted as a function of cos 2θ for the three absorption bands of the reaction centers at 757, 801 and 860 nm, respectively.

Utilizing Eqn. 6 for the slopes of lines drawn through the data points, the angle β between the transition moment and plane of the membrane was determined (Fig. 4B). Each data point represents an average of at least 10 single measurements with three different membranes. The error bars indicate the 95% confidence interval of the data. The slope calculated by linear regression resulted in following values. Two bands gave unique values of the angles: $\beta(\lambda = 757\ \text{nm}) = 29.5 \pm 1.2°$ and $\beta(\lambda = 860\ \text{nm}) = 41.3 \pm 0.9°$. Interpretation of the result of the $\lambda = 801$ nm band is not uniquely possible. The slope is -0.02 ± 0.03 and covers slope 0 which may be the consequence of a random orientation of the chromophore. For further clarification an experiment with a different tilt angle of membrane and incident light beam is required. However, this experiment was not possible with the available membrane cuvettes.

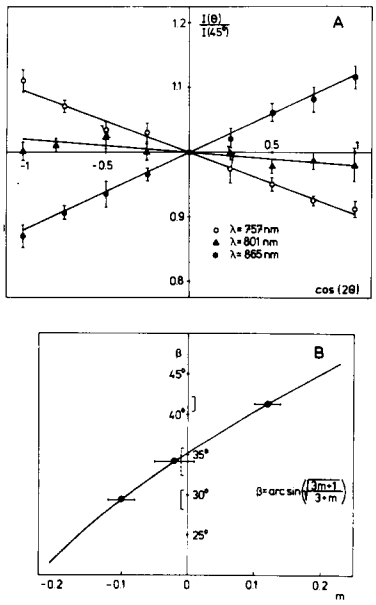


Fig. 4. Photoelectrical effects with linearly polarized light. (A) Ratio of photocurrents as a function of the polarization angle according to Eqn. 6 for the three different chromophores of the reaction center. (B) Correlation between the slope m of the lines of panel A and the angle β between transition moment and membrane plane.

The dichroic ratio of the data is calculated to the $D(860\ \text{nm}) = 1.27 \pm 0.04$, $D(801\ \text{nm}) = 0.97 \pm 0.06$ and $D(757\ \text{nm}) = 0.82 \pm 0.04$. Under the assumption that the chromophore at $\lambda = 801$ nm is not randomly oriented, and that, under illumination with red light ($\lambda > 700$ nm) the three chromophores are excited proportionally to their absorption, an average slope can be caluclated of $+0.003$. The measured average value in red light was 0.0025.

The influence of o-phenanthroline

In order to reduce the possible kinetic processes in the reaction center-containing membrane to an electron transfer only between the donating bacteriochlorophyll dimer and the primary acceptor Q_I, the transition to the secondary acceptor Q_{II} was blocked by *o*-phenanthroline. The inhibition of electron transport by *o*-phenanthroline has been demonstrated in membranes [12,13], chromatophores and isolated reaction centers [26,27]. The experiments described in Refs. 12 and 13 could be confirmed.

Due to the slow back-reaction of the electron from the secondary electron acceptor Q_{II} compared to that from the primary quinone Q_I, the inhibition of electron transfer from Q_I to Q_{II} could be demonstrated using an experiment with and without 0.5 mM *o*-phananthroline. The initial maximum photocurrent of a reaction center- and 5 μM Q_{10}-containing membrane with 1.25 mM potassium ferricyanide was measured under repetitive illumination for 1/4 s every 2 s. For evaluat-

ing the effect in the presence of the blocker a correction for the dark effects had to be made. The results are shown in Fig. 5. In the presence of *o*-phenanthroline the dark period of 1.75 s was sufficiently long to rereduce all the bacteriochlorophyll dimers, so that the signals were of constant, full amplitude. When the electrons were allowed to pass on to the secondary ubuquinone Q_{II} and into the Q pool the dark period was only long enough to rereduce 91% of the available reduced reaction centers before the illumination. After a long dark period the original maximum photocurrent could be obtained again.

The presence of *o*-phenanthroline reduces the possible electron movements according to Fig. 10 to the processes $P \rightarrow Q_I$ with rate constant k_1, and $Q_I^- \rightarrow P^+$ with rate constant k_2. During an illumination, a transient photocurrent is observed which is caused by relaxation of the electron distribution into a new steady state according to k_1 and k_2. After the illumination, the light-driven process $P \rightarrow Q_I$ stops and $k_1 = 0$. The back-reaction to the 'resting' state of the reaction state is controlled only by one rate constant, k_2. In order to exclude artefacts produced by secondary effects by an agent of the type mentioned in Ref. 14, the analysis of the back-reaction had to be done using single, unaveraged data immediately after the addition of the *o*-phenanthroline. In addition to these special experiments the rise time of the current amplifier was increases to 3 ms to reduce membrane noise.

If all other pathways of electron movement are blocked, the back-reaction $Q_I^- \rightarrow P^+$ can be treated as a first-order reaction and the reaction can be described by a single exponential. The analysis of 15 experiments resulted in the finding of a double-exponential process with a fast time constant of 106 ± 14 ms corresponding to a half-time of 70 ms and a slow time constant of 590 ± 150 ms. The ratio of the amplitudes of both processes is $A(\text{fast})/A(\text{slow}) = 6 \pm 1$. The variations of the time constants and amplitudes resulted mainly from the fact that the currents of the back-reaction are rather small (in the range of 10 pA). A typical experiment is shown in Fig. 6. A reasonable interpretation of double-exponential processes instead of only a single-exponential one may be based on an effect described by Vermeglio et al. [26]. They observed that *o*-phenanthroline inhibits the reaction $Q_I^- Q_{II} \rightarrow Q_I Q_{II}^-$ but not the reaction $Q_I^- Q_{II}^- \rightarrow Q_I Q_{II}^{2-}$. Therefore, the decay kinetics after the actinic flash on chromatophores or isolated reaction centers in the presence of *o*-phenanthroline were 85% 'fast' (about 80 ms half-time) and 15% 'slow' (in the range of 1 s). The analysis of the photocurrent during illumination also revealed two time constants independent of the light intensity. The slow one was in the range of 100 ms and had an initial amplitude of 5% of the peak current. The main part of the current could be described by an

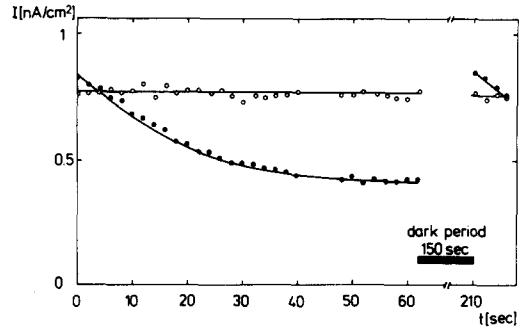


Fig. 5. Behavior of the amplitude of the photocurrent under repetitive illumination (every 2 s for 0.25 s, $\lambda = 850$ nm, 30 mW/cm^2). (●——●) Without *o*-phenanthroline, (○——○) in the presence of 0.5 mM *o*-phenanthroline.

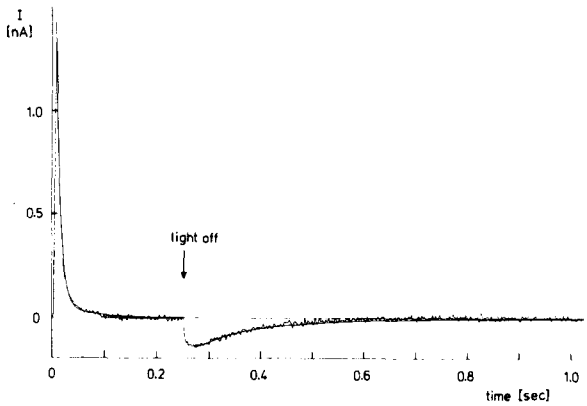


Fig. 6. Photo and dark current of a reaction center-containing lipid bilayer membrane. The line drawn through the data is a two-exponential fit with the following time constants: (photocurrent) $\tau_1 = 9.3$ ms, $\tau_2 = 80$ ms; (dark current) $\tau_1' = 108$ ms, $\tau_2' = 560$ ms. One compartment contained in addition to the electrolyte 1 mM *o*-phenanthroline and 1.25 mM potassium ferricyanide.

exponential function with a time constant of 9.0 ± 0.5 ms.

The integral of the current over the total process, measured from the beginning of the illumination until the end of the back-reaction, was zero within the experimental resolution of the data. This result of a nonexisting net charge transport proved the assumption that the reaction centers are a closed system for electron movements under the given boundary conditions of *o*-phenanthroline blocking and the absence of ferrocytochrome.

The next step was the analysis of the reaction kinetics of the reaction centers with an electron transition from Q_I to Q_{II} with variable Q pool size by adding Q_{10} in concentrations between 5 and 2000 μM to the membrane-forming solution. The photocurrent could be fitted by two exponentials with the time constants of 9.5 ± 1 and 300 ± 50 ms, which were found to be independent of Q_{10} concentration. The initial amplitude of the photocurrent in the presence of Q_{10} increases by a factor of 1.4 compared to currents without Q_{10}. The ratios of the amplitudes of the fast and slow processes increases with increasing Q_{10} concentration from (fast)/(slow) = 4 for no additional Q_{10} to 20 for 200 μM Q_{10}.

The backward current after illumination was also analysed. For no additional Q_{10} or 5 μM Q_{10} in the membrane-forming solution, a backward current was observed with a time constant of 300 ± 50 ms and a maximal amplitude of 10% of the initial photocurrent. For ubiquinone concentrations of 500–2000 μM no backward-directed current was observed in the dark. When the illumination ended before the transient photocurrent became zero the remaining current vanished with a time constant of 10 ms. This behavior indicated a release of the electron out of the reaction centers which was carried by ubiquinones.

Because of the large errors of the single experiments that were attributed to the small amplitudes of the backward-directed current detected without added ubiquinone, a double-illumination experiment as described by Packham et al. [14] was performed. Two light pulses of 16.7 ms duration followed with different dark periods in between. The initial amplitude of the second photocurrent signal can be taken as proportional to the fraction of the reaction centers which have already returned to the starting position of the electron cycle. The dark period between two experiments was 30 s. The ratios of the first and second amplitudes were plotted as a function of the dark period. With more than 600 single measurements on 15 different membranes, and dark periods between 75 and 1000 ms, a single exponential with a time constant of 295 ± 5 ms was obtained and used to describe the recovery of the initial photocurrent. In addition, the temperature was changed. Between 4 and 30°C the time constants varied between 235 ms (30°C) and 330 ms (9°C). Spectroscopic experiments resulted in lifetimes of 60 ms in the absence of Q_{II} or 1 s in the presence of ubiquinone pools [1,14].

The repetition of these double-pulse experiments with Q_{10} in the membrane resulted in a time constant of 1250 ms, which was independent of the concentration of Q_{10} when varied between 500 and 2000 μM in the membrane-forming solution.

The influence of ferrocytochrome c on reaction centers

A number of experiments were performed with cytochrome c_2, isolated from bacteria. A comparison with results obtained with mammalian cytochrome *c* showed no significant difference. Therefore, most experiments were performed with commercial horse heart cytochrome *c*.

The time course of a photosignal of a reaction center-containing membrane in the presence of ferrocytochrome *c* and without additional Q_{10} is displayed in Fig. 2. In the presence of potassium ferricyanide the ratio of peak photocurrents between experiments with and without ferrocytochrome *c* was 2.3 ± 0.3. The total charge moved across the membranes in the presence of ferrocytochrome *c* was determined to be (14 ± 1)-times the charge of the same membranes without ferrocytochrome, while the peak current in the absence of ferrocytochrome *c* appeared 3 ms after the secondary donor was reached after 17 ± 1 ms.

Similar experiments were also conducted in the presence of *o*-phenanthroline, so that only a single turnover per reaction center is possible. Thus, the excited electron moves to the primary acceptor Q_I and the bacteriochlorophyll dimer is reduced by an electron from the ferrocytochrome *c*. The comparison of the peak photocurrents before the addi-

tion of ferrocytochrome *c* and that of the first illumination after the addition showed an increase by a factor 2.0, but the time course of both transients could be described with the same time constant of 10 ms. The second illumination after addition of ferrocytochrome *c*, which followed 5 s after the first, showed no photocurrent.

This experiment could be used to determine the total number of active reaction centers in the membrane. The first illumination after addition of ferrocytochrome *c* resulted in the transfer of an electron in each active reaction center in the membrane. The total charge measured as the time integral of the transient current was calculated to be 0.27 nC/cm^2, a value equivalent to $1.7 \cdot 10^9$ electrons/cm^2.

Because of the absence of a reductase in the bilayer-reaction center system, it is necessary to investigate the process that exchanges the oxidized with the reduced form of cytochrome *c*.

When the peak photocurrent of a membrane containing reaction centers and 200 μM Q_{10} was measured as a function of the ferrocytochrome *c* concentration in one compartment of the membrane cuvette, a saturating behavior was found with a half-saturating concentration of ferrocytochrome *c* at 0.6 μM (Fig. 7). In this multiple-turnover system the peak photocurrent saturates at more than 4-fold greater amplitudes compared to the photocurrent without ferrocytochrome *c*. From investigations of Steinemann and Läuger [27] it is known that the binding of cytochrome on lipid mono- and bilayers is strongly dependent on the ionic strength of the buffer solution. The role of

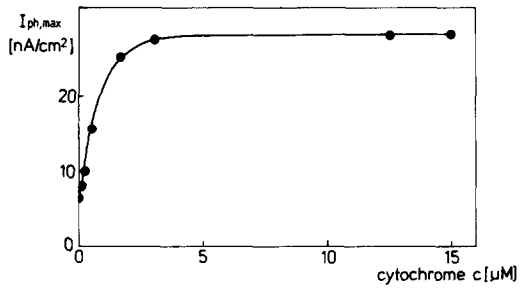


Fig. 7. Peak photocurrent as a function of the ferrocytochrome *c* concentration in buffer (10 mM NaCl, 10 mM Tris, pH 8): each point is the average value of at least 10 single illuminations.

the cytochrome *c* that is partly bound electrostatically on the membrane and partly dissolved in the buffer was analysed by the following experiments.

A membrane containing reaction centers and an excess of Q_{10} was formed in a buffer of 10 mM Tris, pH 8. 12.5 mM ferrocytochrome *c* was added to one side and 250 μM water-soluble Q_0 to the opposite side. The advantage of the ubiquinone without an isoprenoid side chain is its good solubility in water and higher mobility in the membrane, attributed to the missing chain, compared to Q_{10}. The photoresponses of an 8-s illumination were taken for solutions differing in ionic strength between 10 and 1000 mM. The initial peak photocurrent and remaining 'steady-state' current at the end of the illumination were analysed. The results are shown in Fig. 8.

The maximum photocurrent strongly depends on ionic strength at concentrations below 100 mM. This effect parellels the concentration of electrostatically bound cytochrome *c* as indicated by the dashed line taken from the study of Steinemann and Läuger [27] and indicates the concentration of cytochrome *c* bound to a phosphatidylinositol monolayer at corresponding ionic strength (Fig. 8A). The horizontal dotted line gives the peak photocurrent in the absence of cytochrome *c*. At 1 M ionic strength almost no increase in the peak photocurrent is observed.

In contrast to these findings are the results of the remaining steady-state photocurrent in the presence of Q_0 in the membrane and aqueous phase (Fig. 8B). No significant dependence of the current on the ionic strength and on excess Q_{10} could be detected. The dashed line indicates, as in Fig. 8A, the concentration of the membrane-bound cytochrome. The steady-state current could be observed for more than 30 s. At a density of $2 \cdot 10^9$ reaction centers/cm^2, the observed steady-state current of 1.7 nA/cm^2 is equivalent to 5.5 electron turnovers/reaction center per s. In contrast to the peak current, this value is only dependent on the cytochrome *c* concentrations in the solution. Furthermore, the steady-state current was independent of the concentrations of the secondary electron acceptor Q_0 above 10 μM. It is concluded that the reaction centers have access to different reservoirs of the secondary electron donors giving fast and slow rates.

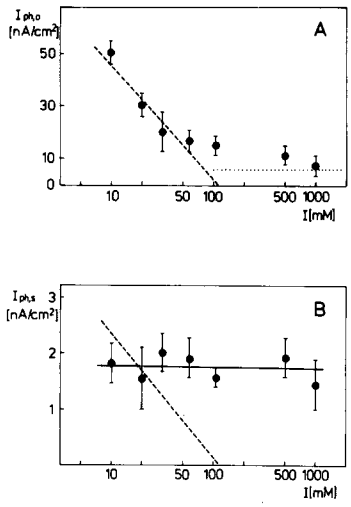


Fig. 8. Maximum (A) and steady-state (B) photocurrent in the presence of the secondary donor (ferrocytochrome *c*) and secondary acceptor (Q_{10} and Q_0) as a function of the ionic strength *I* in the buffer. (A) The dashed line is proportional to the concentration of cytochrome *c* bound to a monolayer, the dotted line indicates the maximum photocurrent in the absence of cytochrome *c*. (B) The solid line is only an eye guide; the dashed line indicates as in A the concentration of the membrane-bound ferrocytochrome *c*.

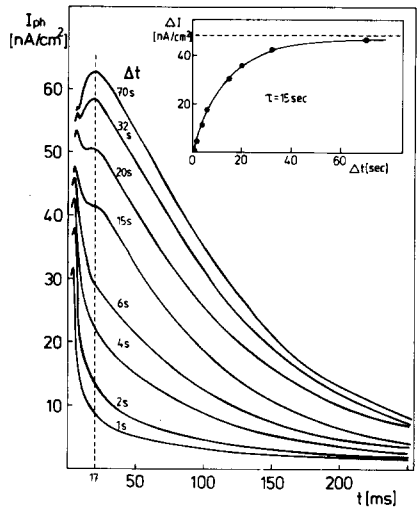


Fig. 9. The photocurrent as a function of the dark period between two illuminations in a system of a reaction center- and Q_{10}-containing lipid bilayer. Asymmetric buffer with 1.25 mM potassium ferricyanide in one phase and 12.5 mM ferrocytochrome *c* in the other aqueous phase. Not displayed is the backward directed dark current which is not significantly different for all photocurrents. The inset shows an analysis of the difference between each photocurrent and the ($\Delta t = 1$ s) current at $t = 17$ ms after the beginning of the illumination.

The process of refilling the cytochrome *c* reservoirs was analysed by measuring the photocurrent as a function of the dark period between illuminations. Before the data were recorded, the sequence 'illumination – dark period' was repeated at least twice to ensure steady-state conditions. The result is shown in Fig. 9. The photocurrent of signals with dark intervals of 1 s exhibited a signal shape of a reaction center/membrane with ferricyanide but in the absence of cytochrome *c*. With increasing duration of the dark period, a new current component appeared with a maximum 17 ms after the beginning of the illumination. This component was isolated by subtracting the signal taken with 1 s dark period from each trace. The increase in this component as a function of the length of the dark period was described by a single exponential with a time constant of 15 s.

Not included in Fig. 9 is the negative, backward-directed dark current, whose maximal amplitude was 19 nA/cm^2 and which did not depend on the length of the dark period between two illuminations.

It was found that the amplitude of the negative dark current was dependent on the time of illumination of the membrane. For illumination times less than 40 ms, no backward current could be observed. When the length of the illumination time was increased a negative backward current could be measured which reached an amplitude of 18% of the peak photocurrent.

Discussion

This paper supports the findings of Schönfeld et al. [12] and Packham et al. [13,14] that a transmembrane movement of electrons can be generated by incorporation of reaction centers in lipid bilayer membranes. It was possible to investigate the properties of pure reaction centers and reaction centers complemented with a ubiquinone pool in the membrane and a ferrocytochrome *c* pool in the aqueous phase.

In contrast to the bacterial membrane, where the reaction centers all face in the same direction, the method of reconstitution used leads to a ran-

dom orientation with half the population producing vectorial electron transport from phase I to phase II and the second half vice versa. Therefore, the membrane by itself shows no net current signal on illumination. To create an asymmetric system, two different methods were used [12,13]. Application of ferrocytochrome to one side provides for one population of the reaction centers present a multiple turnover of electrons in the presence of a ubiquinone pool whereas the population without a secondary electron donor is restricted to a single turnover. In the absence of an electron-acceptor pool, the cytochrome *c* feeds two electrons into the reaction center, thus reducing also the primary donor, the bacteriochlorophyll dimer, and disabling the back-reaction from the electron acceptors. The relaxation of the reaction center to the ground state has a half-lifetime of 16 s (unpublished data). The pathway of the surplus electrons could not be detected, as after 10–15 s the initial photocurrent could be reproduced. The reaction center population that is devoid of cytochrome *c* relaxes in half-times of less than 1 s and can, therefore, be excited by a second illumination following an appropriate delay to the first. The disadvantage of this method is that the observed net signal is the result of two competitive processes and is strongly dependent on the delay between two illuminations. The second way to create an asymmetric membrane system is the addition of potassium ferricyanide to one aqueous phase. Ferricyanide is membrane impermeable and oxidizes the population of reaction centers facing the binding site for cytochrome *c*. Therefore, only the remaining reduced population, aligned in one direction, takes part in the photoreactions. While this conditions makes the data analysis easy, it was uncertain as to whether or not this additional chemical generates artefacts in connection with the reaction center-membrane system.

Therefore, it was proved that the photocurrents observed in a system consisting of a cytochrome/reaction center membrane in the presence of ferricyanide could be generated by the photocurrent of the same system in the absence of ferricyanide plus the photocurrent of a reaction center-containing membrane in the presence of ferricyanide. Fig. 2 shows that, at least qualitatively, this complementary behavior is true. The initial peak photocurrent and the time course for $t > 100$ ms are equal. The decay differs up to 100 ms, but as Fig. 9 shows this part is very sensitive to length of the dark period before the illumination.

A second series of tests monitored the change of the photocurrent in the presence of ferricyanide over the whole lifetime of the membrane. During a period of more than 3 h no drift of the time constants could be observed. As described earlier in this paper, the amplitude of the signal exhibited a drift towards smaller amplitudes. This effect, however, occurred also to the same extent in the absence of ferricyanide and, therefore, the effect must be attributed to other reasons. Working in an oxygen-free atmosphere during the whole experiment had only a small influence on the drift. A flip-flop mechanism of whole reaction centers can be excluded because this effect also appears in the presence of cytochrome *c*, which would reduce the turned-over oxidized reaction center. A reasonable explanation may be a loss of reaction centers which disappeared into the torus of the membrane. It was found that membranes with a large torus showed overall smaller currents and a faster drift of the amplitude. In summary, it can be stated that no effects were observed which suggest any interference that was linked specifically to ferricyanide and the reaction center-membrane system. The data presented in this paper concerning the conditions of the reaction center at the end of the extracting procedure deviated from earlier published findings. Schönfeld et al. [22] reported that in the hexane-extracted reaction centers show only 5% photoactivity in the absence of additional ubiquinone. Packham et al. [14] found larger photocurrents in membrane experiments with octane extracts of reaction center-lipid complexes. However, they reported an almost complete loss of the secondary ubiquinone. The data presented in this paper suggest that at least a part of the reaction centers contains both ubiquinones. The direct analysis of the time course of the back-reaction currents of the reaction center/lipid membranes without additional Q_{10}, as well as the double-illumination data of this system, yield a time constant in the range of 250–300 ms. The addition of Q_{10} not only replaced the missing primary and secondary acceptors, but also generated a pool of free ubiquinone. Therefore, an exchange of semi-

and hydroubiquinone takes place before the observed back-reaction current vanished. The photocurrent increased on average by a factor of 2 (maximum 3.5). However, the time constants did not change. The double-illumination experiments resulted in a time constant of 1.25 s. Only when the electron transfer from Q_I to Q_{II} was blocked by *o*-phenanthroline could the back-reaction Q_I to P be detected. A possible explanation will be described in a later section.

Polarization effects

Polarization effects have to be investigated in planar systems. Up to now no experiments with black lipid bilayers have been published. Rafferty and Clayton [28,29] and Abdourakhmanov et al. [30] investigated the linear dichroism of reaction centers incorporated in dried gelatin films. They published angles of transition moments relative to an axis of symmetry of the reaction center which was aligned to the stretching axis of the gelatin film. Vermeglio and Clayton [31] oriented chromatophores on glass plates by allowing the aqueous suspension to dry. They could determine linear dichroic ratios from the dried films. Tiede et al. [16] deposited densely packed monolayers of reaction centers onto glass plates and found dichroic ratios similar to those stated in Ref. 31.

Comparison of the spectroscopically determined dichroic ratios with the bilayer data reveals a correspondence for the chromophoric group at $\lambda = 760$ nm with values of $D(760\ \text{nm}) = 0.74$ [31], 0.8 [16] and 0.82 (this paper). For $\lambda = 800$–810 nm the bilayer data of 0.97 are between 0.82 [31] and 1.2 [16]. Significant differences can be found between the spectroscopically determined ratios of 1.6 [16,31] and the bilayer data of 1.27 at the absorption band of the bacteriochlorophyll dimer at $\lambda = 860$–870 nm. With respect to the method of taking the bilayer data the discrepancy of only one value cannot be explained. In a comparison of the angles determined for the transition moments published by Rafferty and Clayton [28] an assumption about the axis of symmetry defined in the gelatin experiments has to be made for bilayers to be perpendicular to the membrane plane. Then the results of Rafferty and Clayton [28] can be compared with the angle $\lambda = 90° - \beta$, β being taken from Eqn. 6. Agreement can be found for $\lambda(765\ \text{nm}) = 60.5°$. $\lambda(801\ \text{nm}) = 56°$ and $\lambda(860\ \text{nm}) = 48.7°$ are 12 and 8°, respectively, greater than the angles from Ref. 28. The angle of the transition moment at $\lambda = 801$ nm has to be confirmed because the experimental data could also indicate a random orientation. But, on the basis of angles defined for the other chromophores, it is more probable that the chromophore absorbing at 801 nm also has a fixed angle. An angle in the range of 44° as found in the gelatin film, however, can be excluded. Finally, it must be stated that the different findings are not only based on the insecurity of the definition of the axis of symmetry, but also on the fact that the mathematical treatment that led to Eqns. 2–4 is based on the assumption of an identical angle β for each chromophoric group.

Kinetic analysis of the reaction center

The kinetic properties of bacterial reaction centers have been investigated to a highly advanced level during the past two decades using spectroscopic methods as the main tools. An overview is given in part IV of Ref. 1.

The experiments presented in this paper are discussed in terms of the model derived from spectroscopic investigations. The responses recorded from reaction centers reconstituted in lipid bilayers and their adjacent electron donor, ferrocytochrome *c*, and electron acceptor, the ubiquinone pool, are currents that reflect electron movements with components perpendicular to the membrane plane. Fig. 10 is a schematic diagram of electron pathways with respect to electrogenic properties in accordance with Packham et al. [14]. Those electron transitions with components normal to the membrane plane contribute to the measured current. Therefore, all electron transitions which include the bacteriochlorophyll dimer P as donor or acceptor have to be taken into account. Due to the steric arrangement each normal component could be treated as a movement across half the membrane as a sufficient approximation.

The use of *o*-phenanthroline and donor or acceptor pools allowed setting of the experimental conditions in a way such that only one rate constant was unknown at any given time. The reaction center itself consists of the primary electron donor P and the primary and secondary acceptor Q_I and Q_{II}. The kinetics of this system can be treated

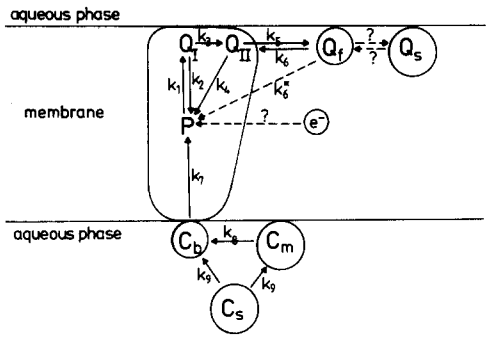


Fig. 10. Diagram of electron pathways in a reaction center incorporated in a black lipid membrane. k_i are the correspondent rate constants. P represents the light-converting bacteriochlorophyll dimer, Q_I the primary electron acceptor, the protein-bound ubiquinone, Q_{II}, the secondary acceptor, which is able to exchange with the ubiquinone pool Q_f and Q_s; subscripts f and s represent fast and slowly accessible, respectively. C_b refers to the ferrocytochrome *c* which is bound to the reaction center, C_m to the membrane-bound ferrocytochrome *c* and c_s to the ferrocytochrome in the aqueous phase.

analytically as shown in Appendix A. The experimental current is calculated to be (Eqn. A13):

$$I = \frac{F}{2}[PQ_IQ_{II}]\left(A_1 e^{-\theta_1 t} + A_2 e^{-\theta_2 t}\right) \tag{7}$$

where A_1, A_2, θ_1 and θ_2 are constant functions of the rate constants. When the electron transition from Q_I to Q_{II} is blocked by *o*-phenanthroline the equation is reduced to a single differential equation and boundary conditions:

$$PQ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} P^+Q^-$$

where $[PQ] + [P^+Q^-] = [RC]$ (RC, reaction center). The solution of the equation:

$$\frac{d[PQ]}{dt} = -k_1[PQ] + k_2([RC] - [PQ]) \tag{8}$$

is well known as:

$$[PQ] = \frac{k_2}{\alpha}[RC] + \left([PQ]_0 - \frac{k_2}{\alpha}[RC]\right)e^{-\alpha t} \tag{9}$$

where $\alpha = k_1 + k_2$ and $[PQ]_0$ is the concentration of [PQ] at time 0.

The solution of Eqn. 8 for the dark back-reaction, $k_1 = 0$, is:

$$[PQ] = [RC] - [P^+Q^-]_0 e^{-k_2 t} \tag{10}$$

where $[P^+Q^-]_0$ is the concentration of 'excited' reaction centers at the end of the illumination. When the system reached equilibrium, then:

$$[P^+Q^-]_0 = [RC] - \frac{k_2}{\alpha}[RC] = \frac{k_1}{\alpha}[RC] \tag{11}$$

According to Eqns. A12 and 11 an expression for the measured currents can be formulated as:

$$I^{p/d} = I_o^{p/d} e^{-\alpha t} \tag{12}$$

For the photocurrent I^p, and for the dark current I^d, where under the assumption of starting from equilibrium conditions I_0 and α are defined as:

$$I_o^p = +\frac{F}{2}k_1[RC] \text{ and } \alpha = k_1 + k_2 \tag{13}$$

for the photocurrents, and:

$$I_0^d = -\frac{k_1 k_2}{k_1 + k_2}\frac{F}{2}[RC] \text{ and } \alpha = k_2 \tag{14}$$

for the dark currents. F if the Faraday constant. The factor $1/2$ must be incorporated because the electron crosses within the reaction center only half the membrane as shown by Packham et al. [14] and as indicated in this paper. With Eqns. 7 and 12 all results of experiments with reaction centers in the absence of donating and accepting electron pools can be used to determine the rate constants and explain the results consistently.

The experimental conditions were chosen in such a way that only a single rate constant had to be determined at any time. The dark current measured in an *o*-phenanthroline-blocked reaction center membrane system allows the determination of k_2, the only rate constant. As mentioned before a 15% component of a slower process was observed that can be explained according to Vermeglio et al. [26] by electron transfers to semiquinones. Because each reaction center belongs to a blocked or nonblocked system, a separation into two populations is possible. Both can be treated separately. The main component of the dark current is controlled by one rate constant k_2 (Eqn.

14). The minor component has two rate constants, according to Eqn. A18, which are $\theta_{1,d} = k_4$ and $\theta_{2,d} = k_2 + k_3$. Under the assumption that $k_2, \theta_{1,d}$ and $\theta_{2,d}$ are not in the same range, which is shown in one of the following paragraphs, k_2 was determined as $9.4 \pm 1.1\ s^{-1}$. k_2 corresponds to a $t_{1/2}$ of 74 ms, which is in agreement with spectroscopic experiments. From the photocurrent the rate constant k_1 can be calculated by using Eqn. 7 and $\alpha = k_1 + k_2$. The average value of α for a light intensity of 30 mW/cm^2 was $111 \pm 6\ s^{-1}$. The value of k_1 then is $101.6 \pm 7\ s^{-1}$. The intensity dependence of k_1 is discussed in one of the following paragraphs.

In the absence of the blocking agent *o*-phenanthroline two more rate constants control the kinetics of the reaction center: k_3 and k_4. If no extra ubiquinone was added a negative back current could be observed that consisted of a single exponential. The rate constant determined was significantly slower than k_2 and, taking Eqn. A18 into account, it must be interpreted as k_4. The nonexistence of the second time constant, $\theta_{2,d}$, indicates that $k_3 \gg k_2$. Two reasons can be given in support: (1) Due to a large k_3 most of the electrons are transferred to Q_{II}, so that the concentration of Q_I is too low to feed an observable signal into the back current. (2) If k_3 is very large compared to k_2, for example, 1000, then $\theta_{2,d}$ is so large that the time needed by the shutter to stop the light is in the same range and thus interferes with the signal. Therefore, the measured rate constant was interpreted as $k_4 = 3.4 \pm 0.4\ s^{-1}$, a result which is supported strongly by the results of the double-illumination experiments, which exhibit also a single exponential with the same rate constant. Eqn. A16 can be taken to estimate the initial back current amplitude when the illumination ends after the system has reached equilibrium. It can be assumed that the fast component is supplied from the $[P^+Q_I^-Q_{II}]$ reservoir, and the slow component from $[P^+Q_IQ_{II}^-]$. The ratio of both gives an upper limit for the ratio of the initial amplitudes $[P^+Q_IQ_{II}^-]/[P^+Q_I^-Q_{II}] = 295$, if k_3 is set at 1000, a value which is reasonable from spectroscopic data [8,32,33].

To obtain information about k_3 the photocurrent was analysed. According to Eqn. A8 $\theta_{1/2}$ was calculated as a function of k_3 with the already determined k_1, k_2 and k_4. It turns out that under the realistic assumption that $k_3 > k_4$ to a first-order approximation, θ_1 is $k_3 f$ with f smaller than 1.2 and that $\theta_2 = 103.5\ s^{-1}$ has a constant value independent of k_3. θ_2 is in good agreement with the experimental results of $105 \pm 10\ s^{-1}$. θ_2 cannot be detected due to the above-mentioned reasons. The small component of the photocurrent with a time constant of 300 ± 50 ms has to include processes with extra-reaction center electron acceptors.

Now the light intensity dependence of the reaction center-membrane system must be discussed. Fig. 4 reveals a limited pool of electrons as the source of the observed currents. If the minor component of the current with a time constant of 300 ms is neglected, the total process is controlled by two time constants, which can be interpreted as $\tau_p = 1/\theta_{2,p}$ for the light-driven photocurrent and $\tau_d = 1/\theta_{1,d}$ for the backward-direction dark current. Therefore, the description of the intensity dependence can be treated with Eqn. 12, where $I_0^p = k_1 \frac{F}{2} [PQ_IQ_{II}]$, the initial peak current is proportional to the initial concentrations of reaction centers with a reduced primary donor P and proportional to the rate constant k_1. α is the observable rate constant $\theta_{2,p}$. In an analogous manner $I_0^d = k_4 \frac{F}{4} [P^+Q_IQ_{II}^-]$, where I_0^d is proportional to the concentration of reaction centers containing an oxidized primary donor P^+; α is k_4.

The net charge, transferred upon illumination and after illumination until a steady state is reached ($I = 0$), can be calculated as:

$$q^p = \int_0^\infty I^p(t)\,dt = I_0^p/\theta_{2,p} \tag{15}$$

$$q^p = \int_0^\infty I^d(t)\,dt = I_0^d/k_4 \tag{16}$$

Correlated values of I_0^p and $\theta_{2,p}$ (respectively, I_0^d and k_4) in Fig. 3 were used to calculated the net charge transferred in both processes. The result was independent of light intensity as suggested by the similar course of data in Fig. 3. The average net charge was calculated to be 0.23 nC/cm^2 for the photocurrent. The net charge for the back-reaction had an average value of 0.22 nC/cm^2. This agreement of both processes underlines the assumption of a reverse process in the reaction

centers with a limited electron pool.

Since $\theta_{2,p}$ and k_2–k_4 are known, the values of k_1 for the different light intensities are calculated from Eqn. A8 and then the total number of reaction centers per cm^2 can be estimated as:

$$n_{RC} = N_A[RC] = \frac{2I_0}{k_1 e_0} \quad (17)$$

From different light intensities a value of $2.9 \cdot 10^9/\text{cm}^2$ was obtained. This reaction center density is of the same order as that determined in the presence of cytochrome *c* and *o*-phenanthroline.

The small current components with time constants greater than 250 ms showed a dependence on the concentration of the ubiquinone pool in the membrane. The reaction centers exchange electrons with those pools. This fact is evident from the 'missing' negative back current in the presence of the Q pool. In Fig. 10 this pathway is denoted by the transition Q_{II} to Q_f with rate constant k_5. Fig. 5 shows a decrease in the initial peak of the photocurrent under repetitive illumination. This decrease can be fitted by a single exponential with a time constant of 22 s. It is not possible to explain this finding with the model of the reaction center as discussed in Appendix A. When the electron is caged in the reaction center a steady state is reached with the second illumination and no further decrease in current amplitude will occur. With the chosen experimental conditions the amplitude depends only on the concentration of the reduced reaction centers. Therefore, electrons have to be removed from the reaction centers. This is, however, a reversible process, since the experiment could be repeated after a longer dark period.

To analyse the reaction center and the interacting pools of electron acceptors and donors a numerical approach was used. In Appendix B the pertinent differential equation system is shown which was integrated by a Runge-Kutta procedure on a computer. The numerical results applied on the reaction center without external electron pools are in complete agreement with those obtained from Appendix A. The double-illumination experiments were performed in a more than a 100-fold excess of Q_{10} in the membrane-forming solution. The concentration of Q_{10} in the membrane could not be measured, so it was suggested that the ratio of reaction center per Q_{10} was the same as that in the solution. The experiments with different Q_{10} concentrations showed no significant dependence of the Q_{10} concentration. With the respect to the long isoprenoid side chain of Q_{10} this effect can be understood by a slow diffusion in the membrane, which maintained the exchange of the reduced Q_{10} at a low level. Slow means in this case that it shows no detectable effect within 5 s. So the electron was fed back again to the reaction center. The data of the double-illumination experiments could not be analysed uniquely. In Fig. 10 is indicated that two possible electron pathways can be envisaged from the so-called fast accessible ubiquinone pool Q_f to the bacteriochlorophyll dimer P^+. Both pathways allow fitting of the data. The uncertainty as to the number of Q_{10} per reaction center increases the set of possible values for k_5, because this step had to be treated as a second-order reaction. Therefore, only the value of k_5 $[Q_f]$ was determined and appropriate values were (100–200)[RC] per s for both pathways. The back-reaction had to be treated as a first-order process, because due to the slow mobility of the Q_{10}, each reduced Q_{10} was exposed only to the donating reaction center. For the different back pathways, the following constants were determined: $k_6 = 120\ \text{s}^{-1}$ and $k_6^* = 0.8\ \text{s}^{-1}$. In both cases k_1–k_4 were used as specified above. An interval of 2 s between two illuminations was sufficient to reach 75–85% of the initial current.

The multiple-illumination experiment in Fig. 5 (in the absence of *o*-phenanthroline) was performed with an excess of 5 Q_{10} per reaction center in the membrane-forming solution. In a double-illumination experiment under these conditions k_6^* was determined to be $1\ \text{s}^{-1}$.

Under repetitive illuminations an exponential decrease in the initial photocurrent was observed, where the nth signal was 90.9% of the $(n-1)$th signal. The net loss of electrons was almost 9% of the active reaction centers per illumination and dark period of 2 s. This part of the electron pathway is indicated in Fig. 10 as a transition to the ubiquinone pool Q_s, which is assumed to be far from the reaction center and only slowly accessible. Another possibility for the recovery of the signal in a sufficiently long-lasting dark period may be an unknown electron-donating source of a membrane chemical.

Collectively, these findings lead to the following kinetic model for the reaction center-electron acceptor interaction: Upon illumination, the absorbed light energy enables an electron to pass to the primary acceptor Q_I and from there, if possible, or if it is existent, to the secondary acceptor Q_{II} and further to the ubiquinone pool, which is close to the reaction center and has a slow exchange rate with the rest of the membrane. From each acceptor a back-reaction is possible, but the rate constants are small when compared to the accompanying forward rate constant. In addition, it was found that the back rate constants decreases with the number of steps that the electron proceeded: $k_2/k_4/k_6^* = 11:4:1$.

The influence of ferrocytochrome c

The inclusion of the secondary electron donor ferrocytochrome *c* enables the reaction center to perform multiple turnovers, and changes the number of electrons in one reaction center as indicated in Appendix B. The photocurrent before and after the addition of ferrocytochrome *c* is plotted in Fig. 2 and the numerical analysis showed that per reaction center an average net transfer of 28 electrons occurred until steady-state conditions were reached.

The investigations of native membranes revealed that the transfer of an electron from a cytochrome to the oxidized bacteriochlorophyll has a typical half-lifetime of 30 μs [9]. The oxidized cytochrome is reduced by a reductase which is situated in the neighborhood of the reaction center. According to Dutton and Prince [9] only two positions of the electron donor are required, functionally 'close' to and 'distant' from the reaction center. This is sufficient to explain the oxidation and reduction mechanism of the cytochrome *c*. The reaction centers reconstituted in lipid bilayer membranes lack a reducing system and have to exchange the oxidized cytochrome *c* with the reduced form within the layer of membrane-bound cytochrome *c* or in the electrolyte.

The experimental results plotted in Fig. 8 show the influence of bound and disolved pools of cytochrome *c*. The analysis of the peak photocurrent shows a dependence on the aqueous concentration of the ferrocytochrome and on the ionic strength of the electrolyte. The analysis of the peak photocurrent and the steady-state current (i.e., the amplitude of the current 8 s after the beginning of illumination) revealed that two different reservoirs of electron donors indeed exist. Both reservoirs could be identified from the experimental evidence. The peak photocurrent is generated by the faster accessible pool, which is dependent on the aqueous concentration of cytochrome *c* and on the ionic strength. Fig. 8A shows that a reasonable agreement exists between the peak photocurrent $I_{ph,0}$ as a function of the ionic strength and the concentration of membrane-bound cytochrome *c*. The cytochrome *c*-binding experiments of Steinemann [27] were performed with monolayers of a negatively charged lipid, because the binding of cytochrome *c* to neutral lipids is smaller by a factor of 20. However, it is reasonable to assume qualitatively the same behavior for a phosphatidylcholine membrane. The steady-state current exhibited a dependence only on the concentration of cytochrome *c* in the electrolyte. This indicates that the membrane-bound cytochrome *c* does not contribute significantly to the steady-state currents. As a consequence, the source of donated electrons can be attributed to the aqueous cytochrome *c* pool.

The experiments shown in Fig. 9 proved that the membrane-bound cytochrome *c* pool can be exhausted, when the illuminations are repeated with short dark periods in the range of 1 s. According to these findings the system of electron pathways is completed as shown in Fig. 10. The rate constant k_7, which controls the transition of an electron from a reaction center-bound cytochrome *c* to the oxidized bacteriochlorophyll dimer could not be determined with the present experiments. It will be evaluated by single-turnover experiments carried out with excitation by laser flashes. Typical values of k_7 from the literature are 23 000 [8]. This value is large compared to those of the other rate constants which are related to the bacteriochlorophyll dimer. Therefore, k_7 is not limiting under any conditions.

The rate constant, k_9, that controls the exchange of free and membrane- or reaction center-bound ferrocytochrome *c*, could be determined from the data contained in Fig. 9. For cytochrome *c* concentrations higher than 3 μM a time constant of 15 s was found to be independent of the aque-

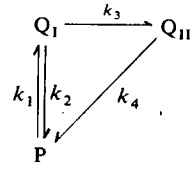
ous cytochrome *c* concentration, so that the exchange with the surface must be limiting. In this case the vaue of k_9 was determined to be 0.07 s^{-1}.

Trials to fit the time dependence of the photocurrents with ferrocytochrome present failed, and had to fail when a rate constant k_8 was assumed which did not take into consideration diffusion phenomena. Parameter sets could be determined which describe the rise in the photocurrent, but they always overestimated the decrease in photocurrent after it reached its maximum value. When the source of the electrons partly is membrane-bound cytochromes, as indicated by Fig. 8a, diffusion will control at least part of the process. A treatment of two-dimensional diffusion on a surface with cylindrical symmetry was published by Adam and Delbrück [34]. From there the diffusion flux at the center can be derived under the boundary condition $c(r = 0) = 0$. This condition, however, cannot be valid. When the illumination lasted longer than 60 ms, a backward directed dark current could be detected. This dark current increases, so that after a 1 s illumination the time integral over the back current resulted in a charge equivalent to 0.6 electrons per reaction center. The time course of the back current revealed at least three exponentials and could be fitted fairly well with rate constants which were in the range of the expected k_2, k_4 and k_6. This fact indicates that 60% of the reaction centers carry electrons on the primary and secondary acceptor which could not be transferred to the ubiquinone pool. Therefore, at each time a certain fraction of the reaction centers cannot accept an electron from the bound cytochrome *c*. Solution of this complicated diffusion problem goes beyond the scope of this paper and has to be treated separately.

The existence of the above-mentioned back current after a longer illumination refers to another point of interest. The observed small steady-state currents, which were measured in the presence of Q_0, cannot be limited by the proliferation of electrons in the reaction centers. On the other hand, the steady-state current does not depend on the concentration of Q_0, when it is available in excess. Therefore, the limiting step must be the exchange reaction of reduced and oxidized Q_{II}.

Appendix A

The reaction centers without ferrocytochrome *c* and without a ubiquinone pool can be treated as a system of three coupled differential equations. The basis is the following reaction scheme:



where only a single electron is transferred. In the following P, Q_I and Q_{II} the concentrations of the three states of the reaction centers: $P \equiv [PQ_IQ_{II}]$, $Q_I \equiv [P^+Q_I^-Q_{II}]$ and $Q_{II} \equiv [P^+Q_IQ_{II}^-]$.
The transition between the three states are determined by the rate constants k_1–k_4 and lead to the differential equations:

$$\frac{dP}{dt} = -k_1P + k_2Q_I + k_4Q_{II} \tag{A1}$$

$$\frac{dQ_I}{dt} = k_1P - k_2Q_I - k_3Q_I \tag{A2}$$

$$\frac{dQ_{II}}{dt} = k_3Q_I - k_4Q_{II} \tag{A3}$$

with the boundary condition:

$$[RC] = P + Q_I + Q_{II} = P_0 \tag{A4}$$

The integration of competitive-consecutive reaction systems can be found elsewhere [35]. Including the additional boundary condition that at the beginning of the illumination $P = P_0$ and $Q_I = Q_{II} = 0$ the solution is:

$$P(t) = P_0\left[1 - \frac{1}{\lambda_1 - \lambda_2}\left(\frac{\lambda_2\kappa'}{\theta_1}(e^{-\theta_1 t} - 1) - \frac{\lambda_1\kappa''}{\theta_2}(e^{-\theta_2 t} - 1)\right)\right] \tag{A5}$$

$$Q_I(t) = -\frac{P_0}{\lambda_1 - \lambda_2}\left[\frac{-\kappa'}{\theta_1}(e^{-\theta_1 t} - 1) + \frac{\kappa''}{\theta_2}(e^{-\theta_2 t} - 1)\right] \tag{A6}$$

$$Q_{II}(t) = -\frac{P_0}{\lambda_1 - \lambda_2}\left[-(\lambda_2 - 1)\frac{\kappa'}{\theta_1}(e^{-\theta_1 t} - 1) + (\lambda_1 - 1)\frac{\kappa''}{\theta_2}(e^{-\theta_2 t} - 1)\right]$$

$$= P_0 - (Q_I(t) + P(t)) \tag{A7}$$

with:

$$\theta_{1/2} = \frac{k_1 + k_2 + k_3 + k_4}{2} \pm \sqrt{\frac{(k_1 - k_2 - k_3 + k_4)^2}{4} + k_1(k_2 - k_4)} \quad \text{(A8)}$$

$$\lambda_1 = \frac{k_2 - k_4}{(k_2 + k_3) - \theta_1} \quad \text{(A9)}$$

$$\lambda_2 = \frac{k_2 - k_4}{(k_2 + k_3) - \theta_2}$$

$$\kappa' = (1 - \lambda_1)k_1 \quad \text{(A10)}$$

$$\kappa'' = (1 - \lambda_2)k_2$$

From this solution the current across the membrane is calculated as the sum of all electron movements perpendicular to the membrane plane:

$$I(t) \propto -\frac{dP(t)}{dt} \quad \text{(A11)}$$

The assumption that the distance between electron donor and acceptor spans half the membrane (Eqn. All) is reformulated:

$$I(t) = -\frac{F}{2}\frac{dP}{dt} = \frac{F}{2}(k_1 P - k_2 Q_I - k_4 Q_{II}) \quad \text{(A12)}$$

where F is the Faraday constant. Using Eqns. A5–A10, the current is given by:

$$I(t) = \frac{FP_0}{2}\left(A_1 e^{-\theta_1 t} + A_2 e^{-\theta_2 t}\right) \quad \text{(A13)}$$

with

$$A_1 = \frac{-\kappa'}{(\lambda_1 - \lambda_2)\theta_1}(k_1\lambda_2 + k_2 + k_4(\lambda_2 - 1))$$

$$A_2 = \frac{\kappa''}{(\lambda_1 - \lambda_2)\theta_2}(k_1\lambda_1 + k_2 + k_4(\lambda_1 - 1))$$

and $A_1 + A_2 = k_1$ fulfills the boundary condition for $t = 0$.

From Eqns. A5–A7 the following equilibrium conditions are derived [35]:

$$Q_I^\infty = \frac{k_1}{k_2 + k_3} P^\infty \quad \text{(A14)}$$

$$Q_{II}^\infty = \frac{k_1 k_3}{k_4(k_2 + k_3)} P^\infty \quad \text{(A15)}$$

$$Q_{II}^\infty = \frac{k_3}{k_4} Q_I^\infty \quad \text{(A16)}$$

Using the boundary condition that $P_0 = Q_I^\infty + Q_{II}^\infty + P^\infty$ then Eqns. A14 and A15 are used to express the equilibrium concentrations in terms of P_0:

$$P^\infty = \frac{k_4(k_2 + k_3)}{k_4(k_2 + k_3) + k_1(k_4 + k_3)} P_0 = \mu_1 P_0$$

$$Q_I^\infty = \frac{k_1 k_4}{k_4(k_2 + k_3) + k_1(k_4 + k_3)} P_0 = \mu_2 P_0 \quad \text{(A17)}$$

$$Q_{II}^\infty = \frac{k_1 k_3}{k_4(k_2 + k_3) + k_1(k_4 + k_3)} P_0 = \mu_3 P_0$$

With Eqn. A17 the back-reaction is calculated from of the equilibrium state under illumination. The result is a current of the form in Eqn. A13 with:

$$A_1 = \frac{\hat{\kappa}'}{(\lambda_1 - \lambda_2)\theta_{1,d}}(k_2 + k_4(\lambda_2 - 1))$$

$$A_2 = \frac{\hat{\kappa}''}{(\lambda_1 - \lambda_2)\theta_{2,d}}(k_2 + k_4(\lambda_1 - 1))$$

with $A_1 + A_2 = -(k_2\mu_2 + k_4\mu_3)$ and:

$$\hat{\kappa}' = k_2\mu_2 - k_4\mu_3 + \lambda_1(k_2 + k_3)\mu_2$$

$$\hat{\kappa}'' = k_2\mu_2 - k_4\mu_3 + \lambda_2(k_2 + k_3)\mu_2$$

The two θ_d result from Eqn. A8 with $k_1 = 0$:

$$\theta_{1,d} = k_4 \quad \text{(A18)}$$

$$\theta_{2,d} = k_2 + k_3$$

Appendix B

The reaction center as a system of complex differential equations interacting with external pools of electron acceptors and donors is either depleted of an electron or carries up to two excess electrons if Q_{II} carries only one electron.

For the reaction center eight different states

have to be defined:

$RC_0 = [PQ_IQ_{II}]$

$RC_1 = [P^+ Q_I^- Q_{II}]$

$RC_2 = [P^+ Q_I Q_{II}^-]$

$RC_3 = [P^+ Q_I Q_{II}]$

$RC_4 = [PQ_I Q_{II}^-]$

$RC_5 = [PQ_I^- Q_{II}]$

$RC_6 = [P^+ Q_I^- Q_{II}^-]$

$RC_7 = [PQ_I^- Q_{II}]$

According to the pathway system for the electrons in Fig. 10 the following equation system has to be integrated:

$$\frac{dRC_0}{dt} = -\left(k_1 + k_6\overline{Q_f}\right)RC_0 + k_1RC_1 + k_4RC_2 + \left(k_8\overline{C_m} + k_9\overline{C_s}\right)RC_3 + k_5Q_fRC_4$$

$$\frac{dRC_1}{dt} = -\left(k_2 + k_3 + k_8\overline{C_m} + k_9\overline{C_s} + k_6\overline{Q_f}\right) \times RC_1 + k_1RC_0 + k_5Q_fRC_6$$

$$\frac{dRC_2}{dt} = -\left(k_4 + k_5Q_f + k_8\overline{C_m} + k_9\overline{C_s}\right) \times RC_2 + k_6\overline{Q_f}RC_3 + k_3RC_1$$

$$\frac{dRC_3}{dt} = -\left(k_6\overline{Q_f} + k_8\overline{C_m} + k_9\overline{C_s}\right)RC_3 + k_5Q_fRC_2$$

$$\frac{dRC_4}{dt} = -\left(k_5Q_f + k_1\right)RC_4 + k_6\overline{Q_f}RC_0 + \left(k_8\overline{C_m} + k_9\overline{C_s}\right)RC_2 + k_3RC_5 + k_2RC_6$$

$$\frac{dRC_5}{dt} = -\left(k_3 + k_6\overline{Q_f}\right)RC_5 + \left(k_8\overline{C_m} + k_9\overline{C_s}\right) \times RC_1 + k_4RC_6 + k_5Q_fRC_7$$

$$\frac{dRC_6}{dt} = -\left(k_5Q_f + k_2 + k_4 + k_8\overline{C_m} + k_9\overline{C_s}\right) \times RC_6 + k_6\overline{Q_f}RC_1 + k_1RC_4$$

$$\frac{dRC_7}{dt} = -k_5Q_fRC_7 + k_6\overline{Q_f}RC_5 + \left(k_8\overline{C_m} + k_9\overline{C_s}\right)RC_6$$

$$\frac{dQ_f}{dt} = -k_5Q_f\left(RC_4 + RC_6 + RC_2 + RC_7\right) + k_6\overline{Q_f}\left(RC_0 + RC_1 + RC_3 + RC_5\right) + k_x\overline{Q_f}Q_s$$

$$\frac{d\overline{C_m}}{dt} = -k_8\overline{C_m}\left(RC_1 + RC_2 + RC_3 + RC_6\right) = k_9C_mC_s$$

Due to the boundary conditions:

$$\sum_{i=0}^{7} RC_i = 1$$

one of the equations (e.g., for RC_7) did not need to be integrated.

In this equation system the electron transition from C_b to P, controlled by the rate constant k_7, is not explicitly formulated. Under the assumption that k_7 is large compared to k_8 and k_1, the transfer of an electron from bound cytochrome to the bacteriochlorophyll is not limiting and, therefore, could be neglected to reduce the number of differential equations.

Further boundary conditions are

$Q_f + \overline{Q_f} = Q_f^{total} = \text{constant}$

$\overline{C_m} + C_m = C_m^{total} = \text{constant}$

$Q_s = \text{constant}$

$C_s = \text{constant}$

Where the concentrations with a bar are the oxidized form, without a bar the reduced form.

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