THE EFFECT OF AN APPLIED ELECTRIC FIELD ON THE CHARGE RECOMBINATION KINETICS IN REACTION CENTERS RECONSTITUTED IN PLANAR LIPID BILAYERS

A. GOPHER,*† Y. BLATT,* M. SCHÖNFELD,* M. Y. OKAMURA,* G. FEHER,*
AND M. MONTAL*‡
Departments of Physics and Biology‡, University of California, San Diego, La Jolla, California*

ABSTRACT Reaction Centers (RCs) from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* were incorporated in planar bilayers made from monolayers derived from liposomes reconstituted with purified RCs. The photocurrents associated with the charge recombination process between the reduced primary quinone (Q_A^-) and the oxidized bacteriochlorophyll donor (D^+) were measured as a function of voltage (-150 mV $< V <$ 150 mV) applied across the bilayer. When Q_A was the native ubiquinone (UQ) the charge recombination was voltage independent. However, when UQ was replaced by anthraquinone (AQ), the recombination time depended on the applied voltage V according to the relation $\tau = 8.5 \times 10^{-3}\, e^{V/0.175} s$. These results were explained by a simple model in which the charge recombination from UQ^- proceeds directly to D^+ while that from AQ occurs via a thermally activated intermediate state, $D^+I^-Q_A$, where I is the intermediate acceptor. The voltage dependence arises from an electric field induced change in the energy gap, ΔG^0, between the states $D^+I^-Q_A$ and $D^+IQ_A^-$. This model is supported by the measured temperature dependence of the charge recombination time, which for RCs with AQ gave a value of $\Delta G^0 = 340 \pm 20$ meV. In contrast, ΔG^0 for RCs with UQ as the primary acceptor, is sufficiently large (~550 meV) so that even in the presence of the field, the direct pathway dominates. The voltage dependence shows that the electron transfer from I^- to Q_A is electrogenic. From a quantitative analysis of the voltage dependence on the recombination rate it was concluded that the component of the distance between I and Q_A along the normal to the membrane is about one-seventh of the thickness of the membrane. This implies that the electron transfer from I to Q contributes at least one-seventh to the potential generated by the charge separation between D^+ and Q_A^-.

INTRODUCTION

The primary photochemical event in bacterial photosynthesis occurs in a membrane-bound bacteriochlorophyll-protein complex called the reaction center (RC), (for a review see reference 1). Absorption of a photon by the RC results in the transfer of an electron from the primary donor, D (a bacteriochlorophyll dimer) through an intermediate acceptor complex I (an interacting bacteriopheophytin and bacteriochlorophyll) (2) to the primary acceptor Q_A, which in *Rhodopseudomonas sphaeroides* is a ubiquinone (UQ). Information obtained from x-ray diffraction (3) antibody labeling (4) and sequencing (5–7) suggests that the RC protein spans the bacterial plasma membrane. The sites that bind the secondary donor, cytochrome c_2 and the acceptor Q_A are thought to be located on opposite sides of the membrane (8, 9). This transmembrane arrangement is responsible for the electric potential generated across the membrane by the photochemical event. According to the chemiosmotic theory, this potential plays a central role in the energy transduction process (10). The electron potential generated by the electron transfer across the membrane may in turn affect the rates of the electron transfer steps associated with the charge separation. This problem is addressed in the present work.

A variety of approaches have been developed to monitor electrical events associated with light induced electron transfer in both chromatophores and reconstituted RC-lipid systems (11–25). Direct measurements (with microelectrodes) of light induced electric currents and potentials in chromatophores have, thus far, not been reported. However, such direct measurements have recently been reported in systems of purified RCs incorporated in planar lipid bilayers (16, 17, 20, 22). These studies demonstrate directly the transduction by RCs of light energy into electric currents and have shown that the electron transfer steps from D to Q_A and from cyt c^{2+} to D^+ are electrogenic. Similar conclusions have been reached previously in studies of the electrochromic (carotenoid) shift in chromatophores (11, 12).

Dr. Gopher's present address is The Weizmann Institute of Science, Isotope Department, Rehovot, Israel.
Dr. Schonfeld's present address is Department of Agricultural Botany, The Hebrew University of Jerusalem, Rehovot, Israel.

In the present study we chose a system of RCs reconstituted into planar lipid bilayers. Planar bilayers have the advantage of being accessible from both sides. Consequently it is easy to apply voltages across the membrane and to measure the kinetics of electron transfer by electrical methods. The bilayers were formed from monolayers (26, 27) that in turn were derived from liposomes reconstituted with RCs (28).

We have investigated the effect of an applied voltage on the kinetics of charge recombination (back reaction) between the primary donor and acceptor of the RC. An exponential dependence between the kinetics and the voltage was found for RCs in which the native primary acceptor, ubiquinone, was replaced by anthraquinone. The results are satisfactorily explained by a model in which the energy levels of the charge-separated states are changed by the applied electric field. The results are consistent with the experiments and the model of Gunner et al., (29, 30) who changed the energy levels by varying the redox potential of the primary acceptor. A preliminary account of this work has been presented (31).

GLOSSARY

RC	reaction center
D	primary electron donor
Q_A	primary electron acceptor
UQ	ubiquinone-10
AQ	anthraquinone
I	intermediary acceptor
LDAO	lauryl dimethylamine oxide
cyt c^{2+}, cyt c^{3+}	reduced and oxidized cytochrome c
$\vec{\mu}$	electric dipole moment
$\hat{\mathbf{n}}$	unit vector perpendicular to membrane surface

EXPERIMENTAL PROCEDURES

Materials

Reaction centers from *Rhodopseudomonas sphaeroides* R-26 were isolated and purified as described previously (1), and stored at −70°C in 10 mM Tris buffer, 10 mM EDTA, 0.025% LDAO (Onyx Chemicals, Jersey City, NJ) pH 8. Soybean lipids, UQ (bovine heart) and cytochrome c (horse heart type VI) were from Sigma Chemical Co. (St. Louis, MO). Phospholipids were purified according to Kagawa and Racker (32). Terbutryne was from Chem Service (Westchester, PA). Anthraquinone (AQ), obtained from Aldrich Chemical Corps. (Milwaukee, WI) was recrystalized twice from methanol.

Removal of UQ from RCs followed by reconstitution with UQ or AQ

UQ was removed from RCs as previously described (33), to a final value of 0.09 UQ per RC. UQ-depleted RCs (2.5 μM) were mixed with either an equimolar amount of UQ (33) or with a five times molar excess of AQ (added from 700 μM ethanolic stock solution) and reconstituted into liposomes, as described later. The final ethanol concentration was always <2%. The amount of quinone equivalents bound per RC was determined spectrophotometrically from the charge recombination kinetics at room temperature, as described previously (34). After reconstitution, the preparations contained either 0.9 UQ per RC or 0.7 AQ per RC.

Incorporation of RCs in Lipid Vesicles

Phospholipids were dispersed in 0.1 M KCl, 10 mM imidazole, pH 7, to a final concentration of 20 mg/ml. 10 nmol of RCs (in 100 μl, 0.025% LDAO) were added to 1 ml of the phospholipid suspension and sonicated until the absorbance reached a constant value ($A_{650} = 0.3$) (~10 min). This procedure is a modification of that previously described (35). Liposomes were stored at 4°C and used within 2 d. The kinetics of charge recombination of RCs in liposomes were measured with a spectrophotometer of local design (36).

Formation of Planar Bilayers

Planar bilayers were formed from monolayers (26, 27). Liposomes, into which RCs were incorporated, were transformed into monolayers at the air-water interface (28). Bilayers were formed by apposing two such monolayers across an aperture (0.2–0.3 mm diameter) in a 12.5 μm thin teflon septum (Yellow Springs Instruments, Yellow Springs, OH) separating two compartments (27). Liposomes containing RCs (see above) (20 mg lipid/ml) were diluted 1 : 20 into 10 mM KCl, 10 mM $CaCl_2$, 10 mM imidazole pH 7, and added to each compartment. To form stable bilayers the surface pressure of the monolayers had to be increased by the addition of 10 mM $CaCl_2$ from 10 to 47 dyn/cm. Surface pressure was measured by the Wilhelmy plate method (37) with an instrument of local design using a sand blasted platinum plate (2 × 1 × 0.05 cm) suspended from a linear displacement electrical transducer (SS 102 GL; Collins Corp., Long Beach, CA). The membrane capacitance and conductance were 0.7–0.74 $\mu F/cm^2$ and 10^{-7}–$10^{-8} S/cm^2$, respectively.

Light Source

The planar bilayer was illuminated through a window in the front compartment with a 50 mW He-Ne laser (λ_{max} = 6328 Å) (Model 125; Spectra-Physics, Inc., Mountain View, CA). The beam passed through a shutter having a 1 ms opening time (model SD-1000; Uniblitz, Rochester, NY). It was enlarged with a beam expander (model 332; Spectra-physics) and the central uniform portion was focused by a lens on the membrane aperture to avoid the generation of photocurrents of RCs adsorbed to the teflon septum (24, 25). The laser power was sufficient to bleach the RCs in less than the opening time of the shutter, (i.e., <1 ms).

Detection of Photocurrent

Two standard calomel electrodes (No. 41239; Beckman Instruments, Inc., Palo Alto, CA), shielded from light, were introduced into the opposing compartments and connected to a variable voltage source. The current was measured with an operational amplifer (LF357H; National Semiconductors, Santa Clara, CA) with a feedback resistor of $10^9\Omega$ and a time constant of 250 μs. The output from the amplifier was displayed on a digital oscilloscope (model 1090; Nicolet Instrument Corp., Madison, WI). All data were taken starting 1 h after formation of the bilayer. The reason for this delay was that the amplitude of the photocurrent increased during the first hour after bilayer formation; thereafter the current remained constant for the lifetime of the bilayer (up to ~10 h).

Experiments were performed at 22 ± 2°C except when otherwise noted. Decay constants, τ, are expressed in terms of the time it takes to reach $(1/e)$ of the initial value.

THEORETICAL CONSIDERATIONS

The Recombination Kinetics in the Absence of an Applied Electric Field

The main electron transfer reactions in RCs under illumination are schematically illustrated in the energy level diagram of Fig. 1. Of particular interest is the charge

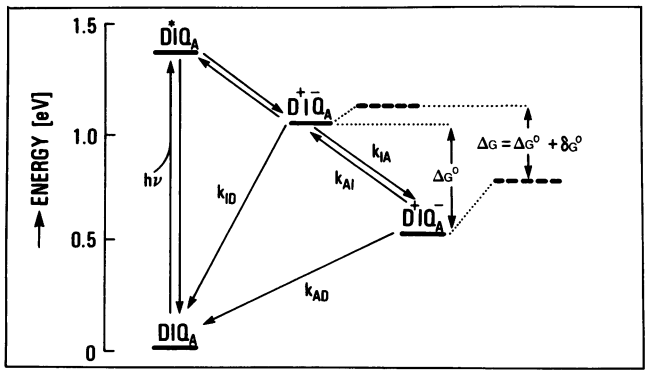

FIGURE 1 Simplified energy level scheme showing electron transfers (*arrows*) in reaction centers of *R. sphaeroides*. The state $D^+IQ_A^-$ can decay either via the direct pathway (with rate k_{AD}) or via the intermediate state $D^+I^-Q_A$, depending on the value of the energy difference, ΔG^0 (Eq. 4). An electric field changes ΔG^0 by δG^0 and affects, therefore, the recombination rate via the indirect pathway (Eq. 11). The change in energy levels, i.e., a reduction in ΔG^0, is drawn for the direction of the electric field as shown in Fig. 2 (according to the convention shown in the inset of Fig. 4, V is negative). When the field is reversed, the energies of the two states are lowered and ΔG^0 is increased.

recombination of the state $D^+IQ_A^-$ since it is accompanied by a current pulse measured in our experiments. The charge recombination from $D^+IQ_A^-$ to DIQ_A can proceed either directly (with rate constant k_{AD}) or via the intermediate state $D^+I^-Q_A$. Which of these pathways dominates at a given temperature will depend on the free energy difference, ΔG^0, between the two states $D^+I^-Q_A$ and $D^+IQ_A^-$. Assuming that the equilibration between these two states is faster than either of the recombination rates, i.e.

$$k_{IA} + k_{AI} \gg k_{AD}, k_{ID}. \tag{1}$$

The two states $D^+I^-Q_A$ and $D^+IQ_A^-$ are in quasiequilibrium and their relative populations are given by the Boltzmann factor

$$[D^+I^-Q_A]/[D^+IQ_A^-] = \exp[-\Delta G^0/(k_bT)], \tag{2}$$

where k_b is Boltzmann's constant and T is the absolute temperature. The fraction α of RCs in state $D^+I^-Q_A$ is given by

$$\alpha = \frac{[D^+I^-Q_A]}{[D^+I^-Q_A] + [D^+IQ_A^-]} = \frac{1}{1 + e^{(\Delta G^0/(k_bT)}}. \tag{3}$$

The observed recombination rate[1] of $D^+IQ_A^-$ is the sum of the direct and indirect rates, i.e.

$$k_{obs} = k_{ID}\alpha + k_{AD}(1 - \alpha) = k_{ID}\left[\frac{1}{1 + e^{(\delta G^0)/(k_bT)}}\right] + k_{AD}\left[\frac{e^{(\Delta G^0)/(k_0T)}}{1 + e^{(\Delta G^0)/(k_bT)}}\right]. \tag{4}$$

[1]Experimentally one measures the characteristic time, τ, (decay to $1/e$ of the initial value), which is the reciprocal of k_{obs}, i.e., $\tau = k_{obs}^{-1}$.

Making the assumption, to be justified later, that

$$e^{\Delta G^0/(k_bT)} \gg 1. \tag{5}$$

Eq. 4 becomes

$$k_{obs} = k_{ID}e^{-\Delta G^0/(k_bT)} + k_{AD}. \tag{6}$$

It is instructive to calculate the energy ΔG^0 which the recombination rates for the direct and indirect paths are equal (i.e., $\alpha = 0.5$). Equating the two terms of the right side of Eq. 6

$$\Delta G^{0*} = k_bT \ln\left[\frac{k_{ID}}{k_{AD}}\right]. \tag{7}$$

At room temperature ($T = 293°K$), $k_bT = 25$ meV; and for RCs with UQ $k_{AD} = 8\ s^{-1}$ and $k_{ID} = 8 \times 10^7\ s^{-1}$ (38). With these values

$$\Delta G^{0*} = 405 \text{ meV}. \tag{8}$$

The energy gap, ΔG^0, in RCs with UQ as the primary acceptor has been determined to be 500–600 meV (29, 39) while for AQ, $\Delta G^0 = 340$ meV (see later section). Since one of these numbers is larger and the other smaller than the critical value of 405 meV (Eq. 8), we anticipate a radically different behavior in these two cases. For

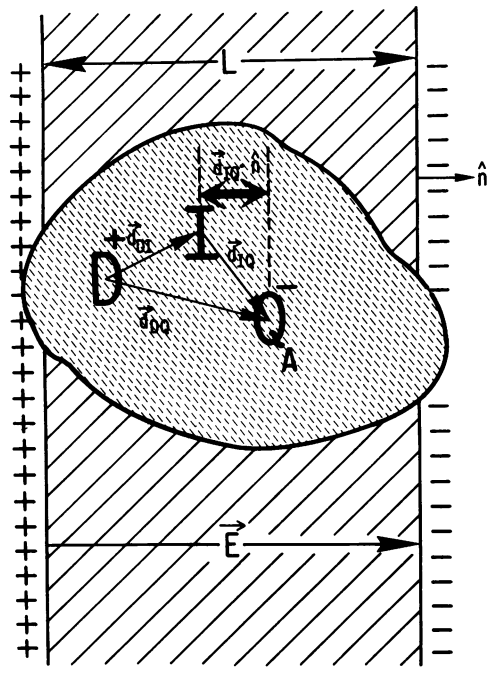

FIGURE 2 Schematic representation of the reactants in RCs with respect to the planar bilayer and the applied electric field, $\vec{E}$. The effect of the electric field on the kinetics of charge recombination arises from the difference in the components of the dipole moments of the states $D^+I^-Q_A^-$ and $D^+IQ_A^-$ (see Fig. 1) along the electric field. This difference is proportional to $\vec{d}_{IQ} \cdot \hat{n}$ (see heavy arrow and Eqs. 9–11). We have made the simplifying assumption that the distances between reactants and their orientations do not change during charge separation. In vivo, the *left* side represents the inside and the *right* side the outside of the chromatophore.

AQ(ΔG^0 = 340 meV), Eq. 6 predicts that ~90% of the recombination proceeds via the indirect pathway,[2] whereas for UQ (ΔG^0 = 550 meV), more than 99% recombine via the direct pathway.

The Effect of an Applied Electric Field on the Charge Recombination Rate

The applied voltage is expected to change the observed kinetics if the charge recombination proceeds via the indirect pathway, involving the rate k_{ID}, (see Fig. 1) and if the energy between the two charge separated states, ΔG^0, is changed. The latter will occur if the components of the dipole moments along the electric field for the two states are different. The situation is schematically illustrated in Fig. 2. The change in energy, ΔG^0 in an applied electric field is given by

$$\Delta G^0 = -(\vec{\mu}_{D^+Q_A^-} - \vec{\mu}_{D^+I^-}) \cdot \vec{E}_{loc} = -q(\vec{d}_{D^+Q_A^-} - \vec{d}_{D^+I^-}) \cdot \vec{E}_{loc} = q\,\vec{d}_{IQ} \cdot \vec{E}_{loc}, \quad (9)$$

where $\vec{\mu}$ are the dipole moments of the charge-separated states, q the electronic charge, $\vec{d}$ the distances between the charges (assumed to be point charges) and $\vec{E}_{loc}$ is the effective field seen by the dipoles. If we make the simplifying assumption that the local field equals the applied field, we obtain

$$\vec{E}_{loc} \simeq \vec{E} = V\hat{\mathbf{n}}/L, \quad (10)$$

where L is the width of the bilayer across which the applied voltage V appears and $\hat{\mathbf{n}}$ is a unit vector normal to the membrane surface (see Fig. 2). Combining Eq. 6, 9, and 10, the recombination rate via the indirect pathway in the presence of an applied field is given by

$$k_{obs} = k_{ID}e^{-\Delta G^0/(k_bT)}\, e^{-\delta G^0/(k_bT)} = k_{ID}e^{-\Delta G^0/(k_0T)}\, e^{-qV(\vec{d}_{IQ}\cdot\hat{\mathbf{n}}/L)/(k_bT)} = k^0_{obs}\, e^{-qV(\vec{\mathbf{d}}_{IQ}\cdot\hat{\mathbf{n}}/L)/(k_bT)}, \quad (11)$$

where k^0_{obs} is the recombination rate in the absence of an applied electric field. We have here assumed that k_{ID} is field independent (see Discussion). Eq. 11 predicts that at room temperature an applied potential of 25 meV would change k_{obs} by 1/e (k_bT = 25 meV) if d_{IQ} were to span the entire membrane. Thus, from the observed voltage dependence of k_{obs} one can estimate the component of d_{IQ} along the normal of the membrane surface.

Determination of the Energy ΔG^0 between $D^+I^-Q_A$ and $D^+IQ_A^-$

When the charge recombination proceeds via the indirect pathway as we believe to be the case at room temperature in RCs in which the native ubiquinone is replaced by anthraquinone, Eq. 6 can be rewritten as

$$\ln k_{obs} = -\Delta G^0/(k_bT) + \ln k_{ID}, \quad (12)$$

where ΔG^0 is the Gibbs free energy $\Delta H^0 - T\Delta S^0$. Eq. 12 can be rewritten in terms of the enthalpy ΔH° and entropy ΔS°

$$\ln k_{obs} = -\Delta H^0/(k_bT) + \Delta S^0/k_b + \ln k_{ID}. \quad (13)$$

Thus, by plotting $\ln k_{obs}$ vs. $1/T$, the value of ΔH° can be obtained from the slope of the line and the value of ΔS° from the intercept of $\ln k_{obs}$ at $1/T \rightarrow 0$.

RESULTS

The Basic Photoresponse in Functionally Oriented RCs

General Considerations. Planar lipid bilayers formed from two identical monolayers with interspersed RCs do not exhibit photocurrents in the absence of exogenous secondary reactants. This is to be expected from the symmetry of the system, i.e., RCs with opposite orientation are inserted into the membrane with equal probability. Consequently, photocurrents with equal amplitudes and opposite directions are generated. No net current is, therefore, observed. The same, rather unexpected, situation prevails when liposomes with RC were introduced into one compartment and only lipids into the other. Apparently, under these conditions, there is also no net orientation of RCs.

To observe a photocurrent, the symmetry of the system has to be broken. RCs pointing in one direction have to be preferentially modified to obtain a net "functional" orientation. This can be achieved, for instance, by introducing secondary reactants into one compartment. We have used cyt c^{2+} to reduce D^+ in those RCs whose donors point towards the compartment into which the cyt c^{2+} had been added (16). Other workers have used Fe $(CN)_6^{3-}$ to selectively oxidize D (17, 20).

Fig. 3 *A* shows the photocurrents generated by RCs with UQ as the primary acceptor in the presence of cyt c^{2+}. The periodic light pulse sequence is indicated at the bottom and a schematic representation of the events in the two RC populations is shown at the top of the figure. When the RCs are first illuminated, both populations undergo a one electron charge separation in opposite directions. This event does not generate a net current. Following the charge separation, D^+ is reduced by cyt c^{2+} in one of the RC populations. The observed photocurrent shows that the reduction of D^+ by cyt c^{2+} is electrogenic as has been observed previously (12, 14, 20, 24, 25). The RCs that were reduced by cyt c^{2+} are in the state DIQ_A^- and are effectively inactivated. They can neither undergo charge recombination when the light is turned off, nor can a charge separation between D and Q_A^- occur when the light

[2]For AQ the values for k_{ID} and k_{AD} (at 295°K) have not been reported. We have assumed them to be the same as for UQ. (See Discussion).

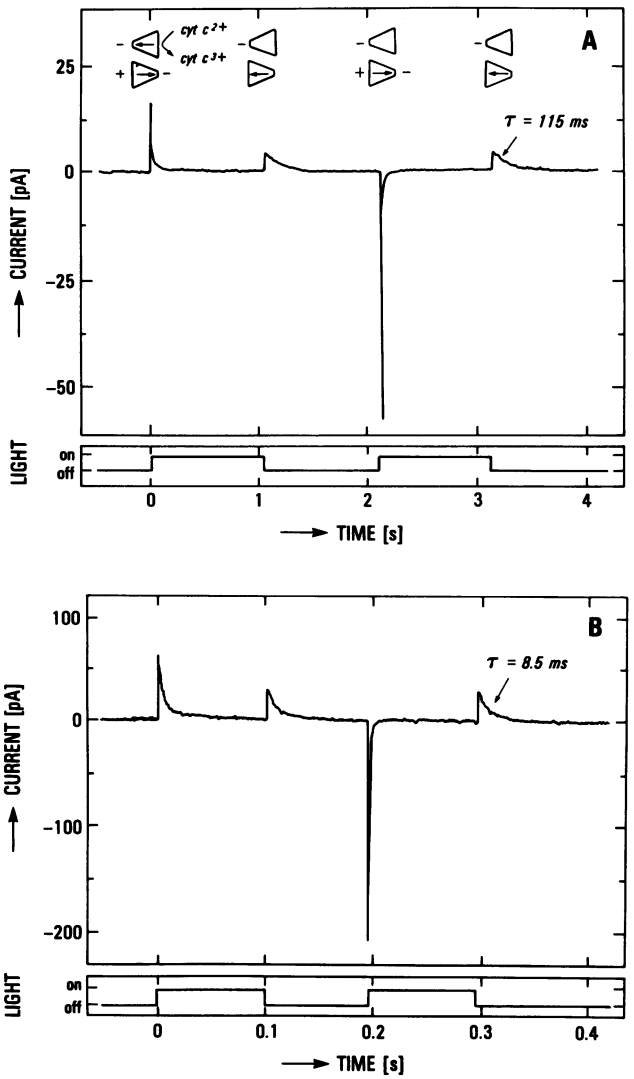

FIGURE 3 Photocurrents generated by RCs incorporated in a planar lipid bilayer. Cyt c^{2+} was added to one side of the bilayer. *Top* (*A*) shows the response of RCs with UQ as the primary acceptor (0.9UQ/RC), *bottom* (*B*) with AQ (0.7AQ/RC). (Note the different time scales in *A* and *B*.) The charge separation events following the periodic light pulses are shown schematically at the *top* of the trace. The photocurrents after the second and all subsequent light pulses are identical but different from the photocurrent observed after the first pulse. The recombination kinetics were obtained from the decay rate of the current after the light was turned off. The values obtained were the same as determined spectrophotometrically with RCs incorporated in liposomes (Fig. 8).

is turned on again. This situation persists as long as the electron is on Q_A^-, i.e., ~5–10 min, the time it takes for the electron on Q_A to leak off. Thus, during that time interval we have a functionally oriented sample and only those RCs that did not interact with cyt c^{2+} are active.

The currents produced at the onset of the second and all subsequent illuminations are due to the charge separation process $DIQ_A \xrightarrow{h\nu} D^+IQ_A^-$, occurring in those RCs whose donors were not reduced by cyt c^{2+}. The time course of the current is given by the rate of build-up of the charge separation, i.e., $d[D^+IQ_A^-]/dt$. In our experiments it was governed by the opening time of the mechanical shutter (1 ms). The current observed when the light is turned off is due to the charge recombination process $D^+IQ_A^- \rightarrow DIQ_A$.

Charge recombination in RCs with UQ as the primary acceptor. The observed charge recombination kinetics in RCs with 0.9 UQ/RC could be fitted with an exponetial having a time constant of 115 ± 5 ms (Fig. 3 *A*), in good agreement with the kinetics determined spectrophotometrically for RCs in detergent solutions (36) and in liposomes (see later section). The time integral of the current varied from membrane to membrane. Since its value is proportional to the number of charges transferred, this finding indicates a variation in the number of RCs in the bilayer. However, the time integrals of the current for the charge separation and recombination were, as expected, the same for each particular bilayer. Typical values for the time integral of the current of 3×10^{-13} Coulombs, corresponding to a density of $\sim 10^{10}$ RCs/cm^2, were obtained.

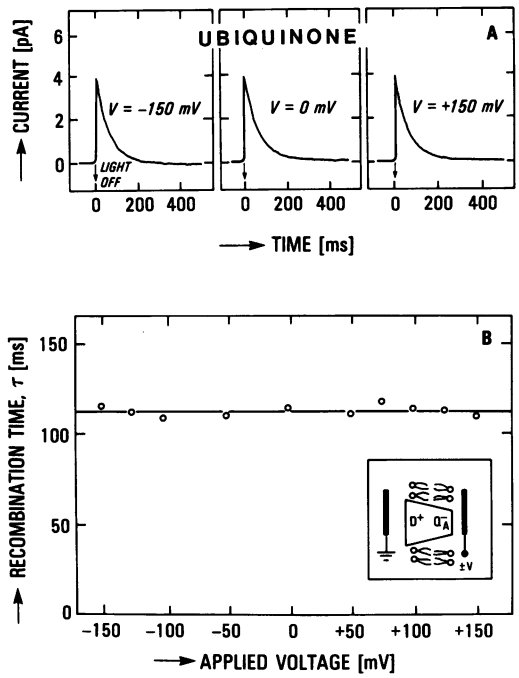

FIGURE 4 The effect of an applied electric field on the kinetics of charge recombination in RCs with UQ as the primary acceptor. RCs with 0.9 UQ/RC were incorporated in a planar bilayer and the current measured in the presence of cyt c^{2+} after 1 s light pulses as described in Fig. 3 (*A*) Time course of current after light pulse is turned off in the absence ($V = 0$) and presence ($V = \pm 150$ mV) of an electric potential in UQ. (*B*) Dependence of the charge recombination rate on the applied voltage. *Inset* shows the polarity of the voltage with respect to the functionally oriented population of RCs. The compartment (*left*) without cyt c^{2+} is defined as being at zero potential. An amplifier that inverts the polarity of the output signal was used.

Charge recombination in RCs with AQ as the primary acceptor. Fig. 3 *B* shows the photocurrents generated by RCs with AQ as the primary acceptor. The results are qualitatively similar to those shown in Fig. 3 *A* (note, however, the different time scale). The charge recombination followed first-order kinetics with a time constant of 8.5 ± 1.0 ms. This value is in good agreement with that found for RCs in detergent solution (29) and in liposomes (see later section). Since excess AQ was required to bind 0.7 AQ/RC, we tested whether some of the AQ was bound to the secondary quinone site. This was accomplished by adding terbutryne, an inhibitor of electron transfer from Q_A to Q_B (40, 41, 44). Addition of 100 μM terbutryne produced no change in amplitude or recombination rate, indicating that AQ was bound only to the Q_A site. Terbutryne was routionely added when RCs with AQ were used as it slowed down the leakage of electrons from Q_A^- to exogenous AQ in the inactivated RC population.

The Effect of an Electric Field on the Kinetics of Charge Recombination

RCs with UQ as the primary acceptor. Fig. 4 shows the kinetics of charge recombination as a function of the voltage applied across the bilayer membrane. Within experimental error (±5%), no change in the kinetics of charge recombination were detected over the entire range of applied voltages (-150 mV $\leq V \leq$ 150 mV).

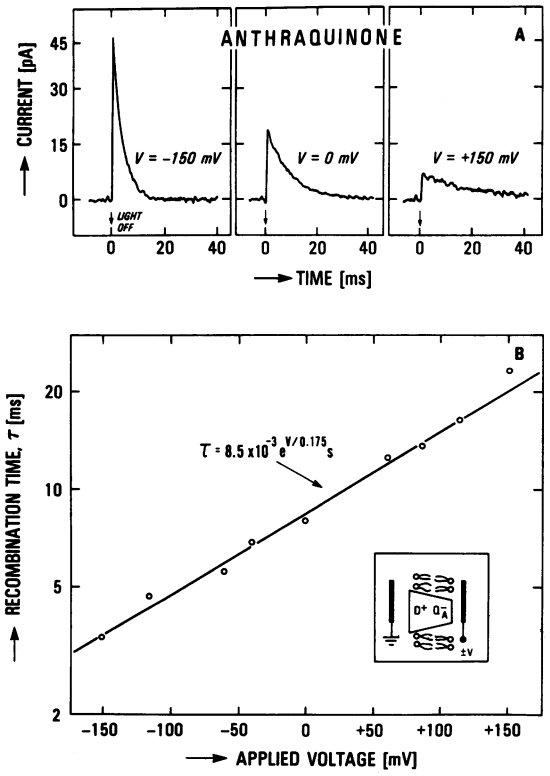

FIGURE 5 The effect of an applied electric field on the kinetics of charge recombination in RCs with AQ as the primary acceptor (0.7 AQ/RC). Solid line represents least-square fit to the data and obeys the relation $\tau = 8.5 \times 10^{-3} e^{V/0.175}$s. Compare the results with those shown in Fig. 4. (Note the difference in time scales and the logarithmic ordinate.)

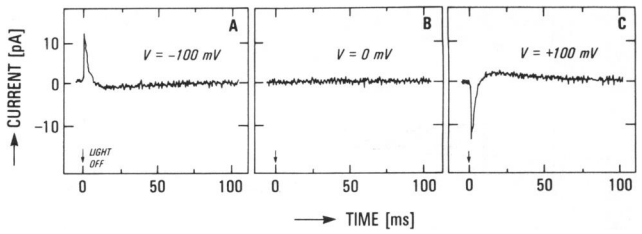

FIGURE 6 Photocurrent obtained from RCs with AQ in the absence of cyt c^{2+}. RCs were incorporated in a planar lipid bilayer and currents measured after a 100-ms light pulse as described in Fig. 3. The current produced in the presence of an electric field (*A, C*) arises from a superposition of two unequal currents that originate from the two RC populations with opposite orientations in the membrane. In the absence of an electric field (*B*) the two currents are equal and cancel each other. Fig. 7 shows the results of a computer simulation of this process.

RCs with AQ as the primary acceptor. When the native UQ was replaced by AQ, a voltage dependent charge recombination was observed (Fig. 5). When the potential was negative in the compartment to which cyt c^{2+} was added, i.e., the side facing the quinone binding site of the active RC population (see inset in Fig. 5 *B*), the recombination rate increased. When the sign of the potential was inverted, a decrease in the recombination rate was observed. The amplitude of the current pulse was found to be inversely proportional to the charge recombination time (Fig. 5 *A, B*). This is to be expected since the integrated area, which represents the number of transferred charges, does not depend on the applied voltage. The dependence of

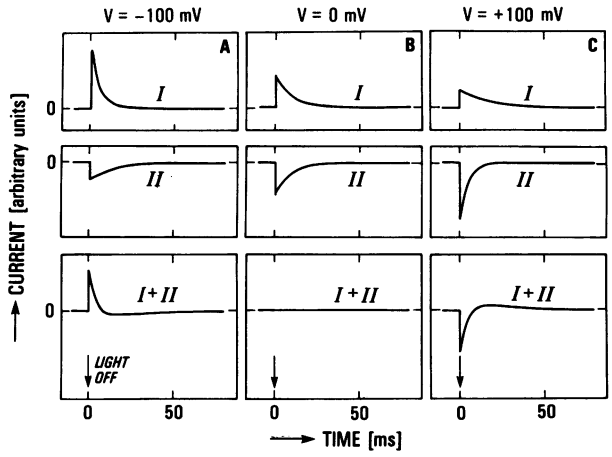

FIGURE 7 Computer simulation of the photocurrents produced by RCs with AQ incorporated in planar lipid bilayers without cyt c^{2+} in the presence of an electric field. Kinetic parameters were taken from experimental results like those shown in Fig. 5. The net current (*lower* panel) is the sum of two currents originating from the two RC populations. Population I is oriented with respect to the field as shown in the inset of Fig. 5 *B*, while population II is oppositely oriented. The shapes of the simulated current pulses (*lower* panel) are in good agreement with the experimentally observed shapes (Fig. 6).

the recombination rate on voltage is shown in Fig. 5 *B*. The data were fitted with an exponential (solid line) of the form:

$$\tau = 1/k_{obs} = 8.5 \times 10^{-3} e^{V/0.175} s. \quad (14)$$

The precision with which the slope was determined was ±5%.

Photocurrents from RCs with AQ in the Absence of Exogenous Reactants

In the beginning of this section we discussed the necessity of imposing an asymmetry on the RC-membrane system to observe a photocurrent. This was accomplished by adding cyt c^{2+} to one compartment. However, for RCs with AQ the addition of cyt c^{2+} is not necessary; the application of an electric field breaks the symmetry and produces an observable current as shown in Fig. 6. The signals are characterized by a fast transient followed by a slower component of opposite polarity that decays to zero. The origin and shape of these signals can be understood by referring to Fig. 5 *A*. When the voltage is negative, half of the RC population will produce a current shown in the left panel of Fig. 5 *A*, while the second half will produce a current of opposite and smaller amplitude decaying with a slower rate as shown in the right panel. The net observed current is the sum of these two contributions. When the voltage is reversed, all currents reverse and a signal of opposite polarity is observed (Fig. 6 *C*). A computer simulation of the signals, using the observed decay times (Fig. 5), is shown in Fig. 7. The bottom three panels are the predicted shapes of the signals. They are in good agreement with the observed currents shown in Fig. 6.

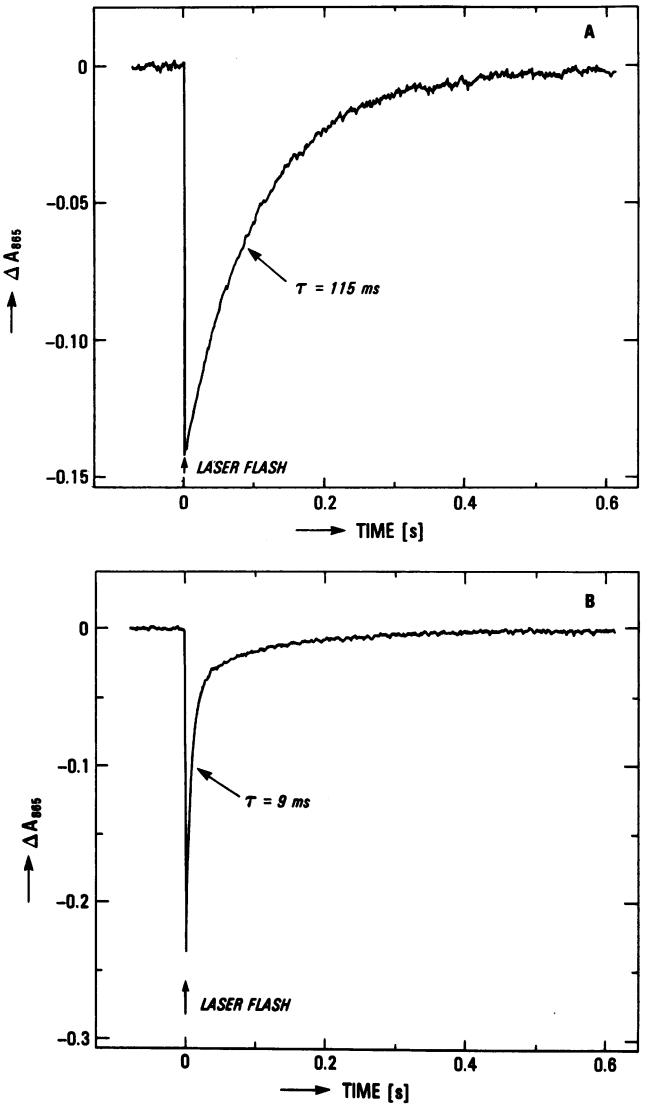

FIGURE 8 Determination of the charge recombination kinetics in RCs reconstituted in liposomes (T = 295°K). The recombination rate was determined from the recovery rate of the optical absorbance change at 865 nm following a single turnover laser flash (*A*) Primary quinone is UQ; RC concentration 1.0 μM with 0.9 UQ/RC. (*B*) Primary quinone is AQ; RC concentration 2.5 μM with 0.7 AQ/RC + 0.1 UQ/RC. The presence of residual UQ accounts for a ~15% slowly decaying component in *B*.

Determination of ΔG^0 in RCs with AQ

As discussed in the previous section, the energy difference, ΔG^0, between the states $D^+I^-Q_A$ and $D^+IQ_A^-$, plays a pivotal role in the recombination kinetics, in particular with respect to the electric field dependence. We wanted, therefore, to determine the value of ΔG^0. This is, technical-

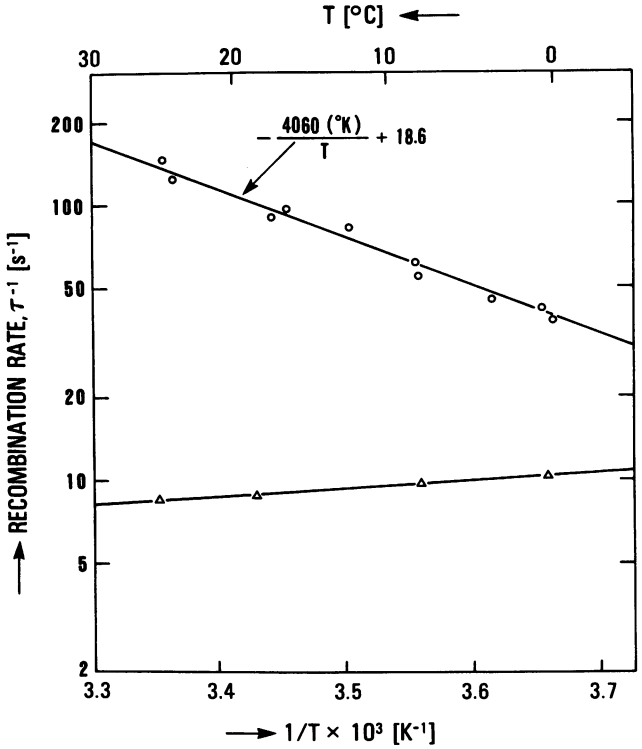

FIGURE 9 Temperature dependence of the charge recombination kinetics in RCs reconstituted in liposomes (see Fig. 8). The primary acceptor was either UQ (△) or AQ (○). The solid lines for the AQ sample represent the theoretical fit: $\ln \tau^{-1} = -4060(K)/T + 18.6$.

ly, difficult to do in planar bilayers; we chose instead a system that closely resembles the planar lipid bilayer. We incorporated RCs into liposomes and measured spectrophotometrically the kinetics of charge recombination following a laser flash. The results are shown in Fig. 8. The recombination time in RCs with UQ was 115 ± 5 ms and with AQ was 9 ± 0.5 ms (T = 295°K). These times are in good agreement with those measured for RCs in planar bilayers (see Fig. 3)

The temperature dependence of the recombination rate is shown in Fig. 9. For RCs with UQ the rate was nearly temperature independent. For RCs with AQ a temperature dependence, characteristic of a thermally activated process (Eq. 12) was observed. The data were fitted with a straight line. From its slope the value for the enthalpy was determined to be $\Delta H^\circ = 350 \pm 20$ meV. From the intercept and the value of $k_{ID} = 8 \times 10^7 s^{-1}$ (38)[3], $T\Delta S^\circ$ was determined at 275°K to be ~10 meV. Thus, the free energy difference $\Delta G^0 = \Delta H^\circ - T\Delta S^\circ = 340 \pm 20$ meV. This is in agreement with the value of 326 meV obtained by Gunner et al. (30) and justifies the assumption made in Eq. 5.

DISCUSSION AND CONCLUSIONS

ATP synthesis in photosynthetic organisms is associated with an electric potential across the plasma membrane (10). The question arises whether this potential affects some of the electron transfer processes that take place in the reaction centers embedded in the membrane. The present work was undertaken to study that question by choosing a simple, well defined, model system. We studied the effect of an electric field on the charge recombination between the primary acceptor Q_A^- and the donor D^+ in reaction centers from *Rhodopseudomonas sphaeroides*. RCs were reconstituted in planar lipid bilayers and the kinetics were determined by measuring the current associated with the charge recombination process $D^+IQ_A^- \rightarrow DIQ_A$ following a light flash. Since RCs were found to orient randomly in the membrane, cyt c^{2+} was added to one side of the membrane to preferentially inactive RCs whose donors pointed towards cyt c^{2+}. This resulted in a functionally oriented system of RCs in the membrane.

The results of the kinetic measurements differed drastically depending whether the primary acceptor was the native ubiquinone (UQ) or whether it had been replaced by anthraquinone (AQ). For RCs with UQ there was no observable effect of the applied voltage (-150 mV $\leq V \leq +150$ mV) on the rate of charge recombination (Fig. 4). In contrast, for RCs with AQ the charge recombination rate depended exponentially on the applied voltage (Fig. 5 and Eq. 15). These results were explained by a model in which the charge recombination proceeds either directly ($D^+IQ_A^- \rightarrow DIQ_A$) or via an intermediate state ($D^+IQ_A^- \rightarrow D^+I^-Q_A \rightarrow DIQ_A$) (see Fig. 1). The value of the free energy difference, ΔG^0, between the states $D^+I^-Q_A$ and $D^+IQ_A^-$ determines whether one or the other process dominates. For $\Delta G^0 > 400$ meV (RCs with UQ) the direct pathway dominates; for $\Delta G^0 < 400$ meV, (RCs with AQ) the charge recombination proceeds via the indirect pathway in a thermally activated process. Since the effect of the electric field is to change ΔG^0, the recombination rate via the indirect pathway will depend exponentially on V (Eq. 11), whereas the direct pathway is unaffected by changes in ΔG^0. This explains the different behavior observed with UQ and AQ reconstituted RCs (compare Figs. 4 and 5).

We now consider the structural implication of these results. For RCs with AQ we found that a transmembrane potential of 175 meV was needed for an e-fold change in the recombination rate (Eq. 15). If I and Q_A were to span the membrane only 25 meV (i.e., k_bT) would be needed. The larger voltage observed means that the component of the distance between I and Q_A along the normal of the membrane surface is $\vec{d}_{IQ} \cdot \hat{n} = (25/175)\ L$ = one-seventh of L where L is the width of the hydrophobic part of the membrane (Fig. 2). With L = 30 Å (26), $\vec{d}_{IQ} \cdot \hat{n} \simeq 4$ Å. The distance d_{IQ} has been estimated to be ~10 Å (43, 44). In view of the simplifying assumptions made (e.g., point charges, neglect of local field effects and the assumption that the entire RC is embedded in the hydrophobic part of the membrane), this should be taken only as a rough estimate. The above results also predict that the electron transfer from I to Q_A should be electrogenic and contribute at least one-seventh to the potential generated by the charge transfer between D^+ and Q_A^-. This is to be compared with the results obtained by Trissl (23) who determined this fraction to be about one-third. The two numbers are comptible if $D^+Q_A^-$ spans only about three-sevenths of the membrane.

Another way of looking at the AQ results is to note that a potential difference of 175 meV across the membrane changes ΔG^0 by 25 meV (i.e. k_BT). We can then estimate the potential needed to observe a field dependent recombination rate in RCs with UQ. For the indirect pathway to become significant, ΔG^0 has to be reduced by ~145 meV (from 550 to 405 meV, see Eq. 8). Assuming that for this case $\vec{d}_{IQ} \cdot \hat{n}$ equals 1/7 of the membrane thickness, the required voltage is -145 mV $\times$ 7 $\simeq -1$ eV. This value exceeds the breakdown voltage of the membrane. It is, of course, possible that an intrinsic field dependence of the direct process may come into play at a lower voltage.

This brings us to the assumptions made throughout this work that within the range of voltages used (-150 mV $\leq V \leq 150$ mV) the direct recombination pathways, k_{ID} and k_{AD} are independent of the applied electric field. That this assumption is justified for k_{AD} is borne out by our finding that RCs with UQ do not show a change in k_{obs} with

[3]See footnote 2.

applied voltage. The electric field does, of course, change the energy level (i.e., redox potential) of $D^+IQ_A^-$ (see Fig. 1). However, this change does not affect the recombination rate. A striking demonstration of this fact is provided by the work of Gunner et al., who reconstituted RCs with quinones having different redox potentials (29). They found that over a wide range of redox potentials k_{obs} remained essentially unchanged; only at low redox potential, when ΔG^0 became sufficiently small (like with AQ), did k_{obs} increase. No experiments have been reported on the field dependence of k_{ID}; we simply assume it to be field independent, in analogy to k_{AD}.

Another assumption that we have made is that k_{ID} for AQ is the same as that measured for UQ. The value of k_{ID} enters logarithmically into the entropy contribution of ΔG^0 (Eq. 13). However, it does not affect the value of $\vec{d}_{IQ} \cdot \hat{n}$ as long as ΔG^0 is small enough to ensure that the charge recombination proceeds via the indirect pathway. That this is the case for AQ is borne out by the experimental results. If, at 295°K in the absence of an applied voltage, part of the recombination pathway were direct, then at lower temperatures and higher applied voltages a larger proportion would decay via the direct pathway (Eq. 6) and k_{obs} would deviate from an exponential dependence on $1/T$ or V. This is not observed (see Figs. 5 *b* and 9). Similarly, the value of k_{AD} for AQ was assumed to be the same as for UQ. It cannot be measured at 295°K for AQ since k_{AD} does not contribute measurably to the charge recombination. At 80°K, k_{AD} for AQ is about five times smaller than for UQ (33). This suggests that the indirect pathway at 295°K for AQ is even more favored than we estimated with the assumption that k_{AD} is the same as for UQ.

In the simplified version of the energy level diagram of Fig. 1 we neglected other possible charge recombination pathways, most notably to the triplet state, denoted P^R (45). An inclusion of these pathways may change the value of k_{ID}, which, however, as discussed in the previous paragraph, does not affect our main conclusions.

What possible bearings do the findings of this paper have on photosynthesis? For photosynthesis to proceed with high quantum efficiency, the back reactions (recombination rates) have to be much slower than the corresponding forward reactions, i.e., the charge separated species have to be stabilized. This is accomplished (like for the state $D^+IQ_A^-$) at an expense of an energy loss ΔG^0. The present work shows how a reduction in ΔG^0 can destabilize the state $D^+IQ_A^-$ by providing an indirect pathway for the charge recombination. Although with AQ the back reaction (8 ms) is still considerably slower than the forward transfer rate to the secondary quinone (~0.1 ms), a further reduction in ΔG^0 below ~300 meV would start to have detrimental consequences on the quantum efficiency. It is also of interest to note that the outside of chromatophores is negative with respect to the inside and the RCs are oriented in the membrane with their donors pointing towards the inside (8). Thus, the membrane potential created during charge separation in vivo decreases ΔG^0 (as depicted in Fig. 1) thereby decreasing the quantum efficiency at high light intensities.

We thank E. Abresch for the preparation of the RCs, R. A. Isaacson for his technical assistance and D. Fredkin, D. Kleinfeld, and W. W. Parson for helpful and stimulating discussions.

The work was supported by grants from the National Science Foundation (PCM 82-02811) and the National Institutes of Health (GM-13191 and EY-02084)

Received for publication 6 December 1984 and in final form 12 April 1985.

REFERENCES

1. Feher, G., and M. Y. Okamura. 1978. Chemical composition and properties of reaction centers. *In* The Photosynthetic Bacteria. W. R. Sistrom and R. K. Clayton, editors. Plenum Publishing Corp., New York. 349–386.
2. Shuvalov, V. A., and W. W. Parson. 1981. Energies and kinetics of radial pairs involving bacteriochlorophyll and bacteriopheophytin in bacterial reaction centers. *Proc. Natl. Acad. Sci. USA.* 78:957–961.
3. Pachence, J. M., P. L. Dutton, and J. K. Blasie. 1979. Structural studies on reconstituted reaction center-phosphatidylcholine membranes. *Biochim. Biophys. Acta.* 548:348–373.
4. Valkirs, G. E., and G. Feher. 1982. Topography of reaction centers subunits in the membrane of the photosynthetic bacterium *Rhodopseudomonas sphaeroides. J. Cell Biol.* 95:179–188.
5. Williams, J. C., L. A. Steiner, R. C. Ogden, M. I. Simon, and G. Feher. 1983. Primary structure of the M subunit of the reaction center from *Rhodopseudomonas sphaeroides. Proc. Natl. Acad. Sci. USA.* 80:6505–6509.
6. Youvan, D. C., E. J. Bylina, M. Alberti, H. Begusch, and J. E. Hearst. 1984. Nucleotide and Deduced Polypeptide Sequences of the Photosynthetic Reaction-Center, B870 Antenna, and Flanking Polypeptides from *R. capsulata. Cell.* 37:949–957.
7. Williams, J. C., L. A. Steiner, G. Feher, M. I. Simon. 1984. Primary structure of the L subunit of the reaction center from *Rhodopseudomonas sphaeroides. Proc. Natl. Acad. Sci. USA.* 81:7303–7307.
8. Prince, R. C., A. Baccarini-Melandri, G. A. Hauska, B. A. Melandri, and A. R. Crofts. 1975. Asymmetry of energy transducing membrane the location of cytochrome c_2 in *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata. Biochim. Biophys. Acta.* 387:212–227.
9. Prince, R. C., R. J. Cogdell, and A. R. Crofts. 1974. Light induced proton uptake by bacterial photochemical reaction centers. *Biochem. Soc. Trans.* 2:162–164.
10. Mitchell, P. 1968. Chemiosmotic coupling and energy transduction. Glynn Research, Bodmin, Cornwall, United Kingdom. 111 pp.
11. Jackson, J. B., and A. R. Crofts. 1969. The high energy state in chromatophores from *Rhodopseudomonas sphaeroides. FEBS (Fed. Eur. Biochem. Soc.) Lett.* 4:185–189.
12. Jackson, J. B., and P. L. Dutton, 1973. The kinetic and redox potentiometric resolution of the carotenoid shift in *Rhodopseudomonas sphaeroides* chromatophores: their relationship to electric field alterations in electron transport and energy coupling. *Biochim. Biophys. Acta.* 325:102–113.
13. Crofts, A. R., and C. A. Wright. 1971. Energy conservation in the photochemical reactions of photosynthesis and its relation to delayed fluorescence. *FEBS (Feb. Eur. Biochem. Soc.) Lett.* 15:89–100.

14. Barsky, E. L., L. Dancshazy, L. A. Drachev, M. D. Ilina, A. A. Jasaitis, A. A. Kordiashim, V. D. Samuilov, and V. P. Skulachev. 1976. Reconstitution of biological molecular generators of electric current. *J. Biol. Chem.* 251:7066–7071.
15. Jursinic, P., Govindjee, and C. A. Wraight, 1978. Membrane potential and microsecond to millisecond delayed light emission after a single excitation flash in isolated chloroplasts. *Photochem. Photobiol.* 27:61–71.
16. Schönfeld, M., M. Montal, and G. Feher. 1979. Functional reconstitution of photosynthetic reaction centers in planar lipid bilayers. *Proc. Natl. Acad. Sci. USA.* 76:6351–6355.
17. Packham, N. K., C. Packham, P. Mueller, D. M. Tiede and P. L. Dutton. 1980. Reconstitution of photochemically-active centers in planar phospholipid membranes: light induced electric currents under voltage clamped conditions. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 110:101–106.
18. Borisov, A. Yu., V. I. Godik, E. A. Kotova, and V. D. Samuilov. 1980. Membrane potential effect on nanosecond recombination luminescende in *Rhodospirilum rubrum. FEBS (Fed. Eur. Biochem. Soc.) Lett.* 119:121–124.
19. Drachev, L. A., A. Y. Semenov, V. P. Skulachev, I. A. Smirnova, S. K. Chamorovsky, A. A. Kononenko, A. B. Rubin, and N. Y. Uspenskaya. 1981. Fast Stages of photoelectric process in biological membranes. III. Bacterial photosynthetic redox system. *Eur. J. Biochem.* 117:483–489.
20. Packham, N. K., P. L. Dutton, and P. Mueller. 1982. Photoelectric currents across planar bilayer membranes containing bacterial reaction centers. Response under conditions of single electron turnover. *Biophys. J.* 37:465–473.
21. Tiede, D. M., P. Mueller, and P. L. Dutton. 1982. Spectrophotometric and voltage clamp characterization of monolayers of bacterial photosynthetic reaction centers. *Biochim. Biophys. Acta.* 681:191–201.
22. Appell, H.J.M. Snozzi, and R. Bachofen. 1983. Kinetic analysis of bacterial reaction centers reconstituted in lipid bilayers. *Biochim. Biophys. Acta.* 724:258–277.
23. Trissl, H. W. 1983. Spatial correlation between primary redox components in reaction centers of *Rhodopseudomonas sphaeroides* measured by two electrical methods in nanosecond range. *Proc. Natl. Acad. Sci. USA.* 80:7173–7177.
24. Blatt, Y., A. Gopher, M. Montal, and G. Feher. 1983. Photovoltages from reaction centers incorporated in interfacial lipid layers. *Biophys J.* 41 (2, pt. 2): 121*a*. (Abstr.)
26. Montal, M., and P. Mueller. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc Natl. Acad. Sci. USA.* 69:3561–3566.
27. Montal, M. 1974. Formation of bimolecular membranes from lipid monolayers. *Methods Enzymol.* 32:545–554.
28. Schindler, H. 1980. Formation of planar bilayer from artificial or native membrane vesicles. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 122: 77–79.
29. Gunner, M. R., D. M. Tiede, R. C. Prince, and P. L. Dutton. 1982. Quinones as prosthetic groups in membrane electron transfer proteins. I. Systematic replacement of the primary ubiquinone of photochemical reaction centers with other quinones. *In* Function of Quinones in Energy Conserving Systems. B. L. Trumpower, editor. Academic Press, Inc., New York. 265–269.
30. Gunner, M. R., Y. Liang, D. K. Nagus, R. M. Hochstrasser, and P. L. Dutton. 1982. Variation in rates of electron transfer in photosynthetic reaction centers with the primary ubiquinone substituted with other quinones. *Biophys. J.* 37 (2, pt. 2): 226*a*. (Abstr.)
31. Gopher, A., Y. Blatt, M. Y. Okamura, G. Feher, and M. Montal. 1983. The effect of transmembrane potentials on electron transfer in reaction centers incorporated in planar bilayers. *Biophys. J.* 41 (2, pt. 2): 121*a*. (Abstr.)
32. Kagawa, Y., and E. Racker. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation: XXV. Reconstitution of vesicles catalyzing $^{32}P_i$-ATP exchange. *J. Biol. Chem.* 246:5477–5487.
33. Okamura, M. Y., R. A. Isaacson, and G. Feher. 1975. Primary acceptor in bacterial photosynthesis: Obligatory role of ubiquinone in photoactive reaction centers of *Rhodopseudomonas sphaeroides. Proc. Natl. Acad. Sci. USA.* 72:3491–3495.
34. Okamura, M. Y., R. J. Debus, D. Kleinfeld, and G. Feher. 1982. Quinone binding sites in reaction centers from photosynthetic bacteria. *In* Function of Quinones in Energy Conserving Systems. B. L. Trumpower, editor. Academic Press, Inc., New York. 299–317.
35. Schönfeld, M., M. Montal, and G. Feher. 1980. Reaction center-phospholipid complex in organic solvents: formation and properties. *Biochemistry.* 19:1535–1542.
36. Kleinfeld, D., M. Y. Okamura, and G. Feher. 1984. Electron transfer in reaction centers of *Rhodopseudomonas sphaeroides.* I. Determination of the charge recombination pathway of $D^+Q_AQ^-$ and free energy and kinetic relations between $Q_A^-Q_B$ and $Q_AQ_B^-$. *Biochim. Biophys. Acta.* 766:126–140.
37. Gaines, G. L., Jr. 1966. Insoluble Monolayers at Liquid-Gas Interfaces. John Wiley & Sons, Inc., N.Y. 386 pp.
38. Parson, W. W., and B. Ke. 1982. Primary photochemical reactions. *In* Photosynthesis. Vol. I. Govindjee, editor. Academic Press, Inc., New York. 331–385.
39. Arata, H., and W. W. Parson. 1981. Delayed fluorescence from *Rhodopseudomonas sphaeroides* reaction centers: Enthalpy and free energy changes accompanying electron transfer from P870 to quinones. *Biochim. Biophys. Acta.* 638:201–209.
40. Wraight, C. A. 1981. Oxidation-reduction physical chemistry of the acceptor quinone complex in bacterial photosynthetic reaction centers: evidence for a new model of herbicide activity. *Israel J. Chem.* 21:348.
41. Okamura, M. Y. 1984. On the herbicide binding site in bacterial reaction centers. *In* Biosynthesis of the Photosynthetic Apparatus: Molecular Biology, Development and Regulation. J. Philip Thornber, L. Andrew Staehlin, and Richard B. Hallick, editors. Alan R. Liss, Inc., New York. 381–390.
42. Okamura, M. Y., R. A. Isaacson, and G. Feher. 1979. Spectroscopic and kinetic properties of the transient intermediate acceptor in reaction centers of *Rhodopseudomonas sphaeroides. Biochim. Biophys. Acta.* 546:394–417.
43. Peters, K., Ph. Avouris, P. M. Rentzepis. 1978. Picosecond dynamics of primary electron-transfer processes in bacterial photosynthesis. *Biophys. J.* 23:207–217.
44. Stein, R. R., A. L. Castellvi, J. P. Bogacz, and C. A. Wraight. 1984. Herbicide-quinone competition in the acceptor complex of photosynthetic reaction centers from *Rhodopseudomonas sphaeroides:* A bacterial model for PS-II-herbicide activity in plants. *J. Cell. Biochem.* 24:243–259.
45. Schenck, C. C., R. E. Blankenship, and W. W. Parson. 1982. Radical-pair decay kinetics. Triplet yields and delayed fluorescence from bacterial reaction centers. *Biochim. Biophys. Acta.* 680:44–59.