RECONSTITUTION DE PHOTOCHEMICALLY ACTIVE REACTION CENTERS IN PLANAR PHOSPHOLIPID MEMBRANES

Light-induced electrical currents under voltage-clamped conditions

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Received 20 November 1979

1. Introduction

The initial light-activated electron transfer occurring within the photosynthetic bacterial reaction center protein (RC) of *Rhodopseudomonas sphaeroides* generates a charge separation and a redox potential difference between an oxidized cytochrome *c* and a reduced quinone. This is summarized in the scheme below in which ferri/ferrocytochrome *c* is the water-soluble cytochrome c_2 that serves as an electron donor to the reaction center; $(BChl)_2$ is a bacteriochlorophyll dimer. BPh is bacteriopheophytin, and Q_I and Q_{II} are the reaction center primary and secondary quinones, respectively. The times given are halftimes. See [1,2] for recent reviews of the reaction center photochemistry.

	Ferrocytochrome *c* $(BChl)_2$ BPh Q_I Q_{II}
1	↓ $h\nu$
	Ferrocytochrome *c* $(BChl)_2$ *BPh Q_I Q_{II}
2	↓ <10 ps
	Ferrocytochrome *c* $(BChl)_2^+$ BPh^- Q_I Q_{II}
3	↓ ~150 ps
	Ferrocytochrome *c* $(BChl)_2^+$ BPh Q_I^- Q_{II}
4	↓ usually <100 μs
	+Ferricytochrome *c* $(BChl)_2$ BPh Q_I^- Q_{II}
5	↓ ~100 μs
	+Ferricytochrome *c* $(BChl)_2$ BPh Q_I Q_{II}^-

Scheme 1

In vivo the reactions of scheme 1 are considered to be directed across the photosynthetic membrane, cytochrome c_2 being on one side of the membrane [3] and proton binding to Q_{II}^- [4,5] occurring on the other. The electrogenic nature of the charge separation has been demonstrated by making use of a variety of indirect methods such as the carotenoid bandshift [6–10], oxidation–reduction poise shifts between $(BChl)_2$ and cytochrome c_2 [10], delayed light emission [11], or externally added oxonol dyes [12,13] as indicators of the light-generated membrane potential. A more direct approach was introduced in [14,15] measuring membrane potentials across preformed planar phospholipid membranes to which were added liposomes incorporating reaction centers (RC); however, the overall geometry of these membranes is not certain.

Planar phospholipid membranes formed from alkane solutions have proven to be useful models for the hydrocarbon matrix of biological membrane [16]. Hydrophyllic proteins were first incorporated into alkane solvents in [17] using phospholipids to carry the protein into solution. This approach has been used to incorporate into alkanes several hydrophobic proteins, including rhodopsin [18], cytochrome oxidase [19], and photosynthetic RCs [20,21] with successful retention of enzymatic activity. Here we describe the flash-induced electron transfer reactions in RCs from *Rps. sphaeroides* solubilized in phospholipid–octane, and report direct measurements of electric currents promoted by the RCs incorporated into planar membranes and bilayers.

2. Materials and methods

Bovine brain phosphatidylserine (PS) was prepared

following the method in [22], egg phosphatidylcholine (PC) was prepared as in [23], while egg phosphatidylethanolamine (PE) was obtained from Supelco, Bellefonte, PA. RCs from *Rps. sphaeroides* strain R26 were isolated based on methods using the detergent lauryldimethylamine-*N*-oxide (LDAO) [24,25]. The RC concentration was determined spectrophotometrically [26,27].

RCs were extracted from the 10 mM Tris–HCl (pH 8), 0.1% LDAO solution into *n*-octane as follows. RCs (0.3–0.5 mg protein equiv.) were added to a presonicated phospholipid dispersion (10 mg total: PC/PE/PS, 3:3:1, by wt). Following a further sonication for 15 min in a water bath sonicator, 100 μl 1 M $CaCl_2$ and 2 ml octane were added and the mixture vigorously stirred for 4 min. The mixture was centrifuged to yield an optically-clear upper octane phase containing RCs and phospholipids. Typically ~85% of the phospholipid [28] and 80% of the protein [29] is extracted into the octane. The octane extract was then used again to extract another batch of RC–phospholipid dispersion. After extracting 4 batches and reducing the amount of octane by evaporation with a stream of nitrogen the procedure yielded 0.5 ml 5–10 μM RC–phospholipid–octane solution. The extraction procedures were performed at room temperature.

Planar membranes were formed by depositing an aliquot of the RC–phospholipid–octane solution over an orifice in a Teflon chamber separating two aqueous phases of distilled water [30]. The lipid films thinned to form uniform membranes that, depending on the preparation, were either black (BLM) or silvery to reflected light and typically displayed membrane resistances of 10^7–10^8 Ωcm^2. There was no consistent difference in the signals exhibited by the two kinds of membranes in the experiments presented here. Electrical signals were monitored by a voltage clamp as described elsewhere. The membranes were illuminated using either a steady-state source (tungsten or mercury arc) or light pulses from a xenon arc or a Q-switched ruby laser directed onto the membrane by a light-guide. Interference filters were used to record the action spectrum (fig.1) of the peak current response to steady-state illumination. Laser or xenon flash activated kinetic measurements on the RC solutions were done spectrophotometrically as in [27]. Ubiquinone-10 and horse heart cytochrome *c* (type III) were obtained from Sigma (St Louis, MO).

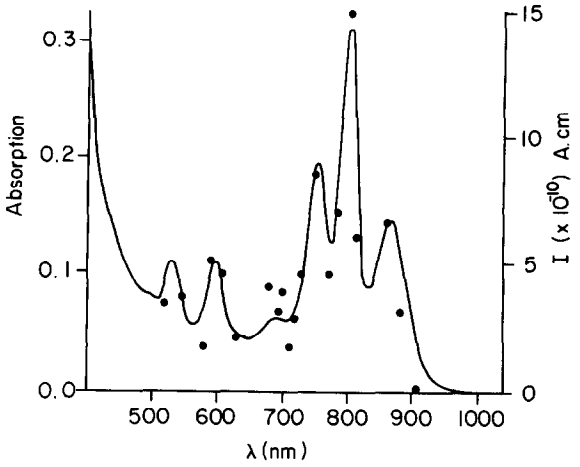

Fig.1. Absorption spectrum of a reaction center–phospholipid–octane solution compared to the action spectrum (•) generated by measurement of the peak current response following steady state illumination of planar reaction center–ubiquinone–phospholipid membranes supplemented with 25 μM ferrocytochrome *c* on one side. Conditions as in fig.3C.

3. Results

3.1. *The absorption properties of RC-phospholipid-octane solution*

Figure 1 shows the absorption spectrum of the RC–phospholipid–octane solution. It closely resembles the spectrum for RCs in aqueous-detergent (10 mM Tris–HCl, 0.1% LDAO) and agrees well with those published for RC–phospholipids in hexane [20,21].

3.2. *The kinetic competence of the RCs in the phospholipid–octane solution*

Figure 2A shows the kinetics of flash-induced reaction center $(BChl)_2$ oxidation and subsequent dark re-reduction. The absorption decrease at 605 minus 540 nm immediately following the flash, results from the oxidation of $(BChl)_2$ (<10 ps; see scheme 1). The subsequent course of $(BChl)_2^+$ re-reduction in the dark displays the very much slower halftime of 75 ms, which is similar to the halftime of return of an electron to $(BChl)_2^+$ from Q_I^- measured in RCs in aqueous-detergent media. This back reaction [1] is readily observed in RCs that are devoid of both Q_{II} and ferrocytochrome *c* which eliminate competing forward reaction steps 4 and 5, respectively, in scheme 1. Thus we tentatively conclude that the RCs in the phospholipid–

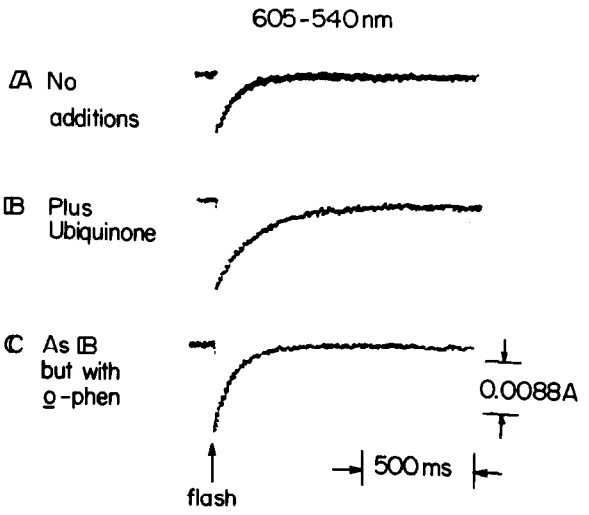

Fig.2. The flash-induced reaction center $(BChl)_2$ oxidation and dark re-reduction in phospholipid–octane solution. The reaction center was 0.6 μM. (A) No additions. (B) As (A) but 300 μM ubiquinone-10 added. (C) As (B) but 5 mM *o*-phenanthroline added.

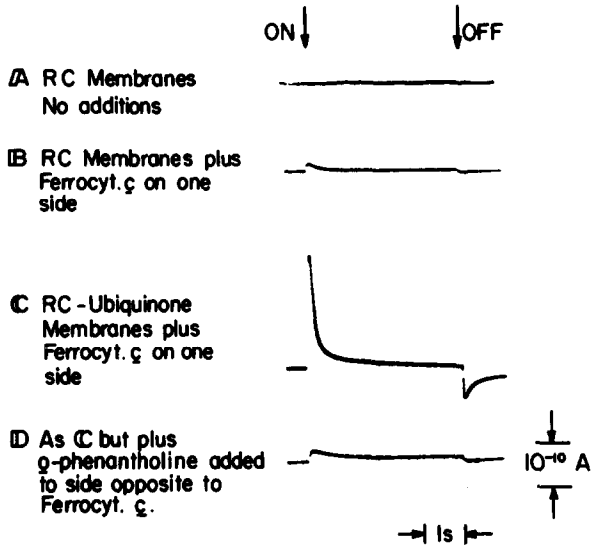

Fig.3. Steady state light-induced electrical currents across the reaction center–phospholipid membranes. The RC in the RC–phospholipid–octane solution from which the membranes were formed was 8.3 μM. (A) RC membrane alone. (B) As (A) but with 25 μM ferrocytochrome *c* added to the aqueous phase of one side of the membrane. (C) As (B) but with 180 μM ubiquinone-10 added to the RC–phospholipid–octane solution before membrane formation. (D) As (C) but with 5 mM *o*-phenanthroline added to the aqueous side opposite the ferrocytochrome *c*.

octane solution display unaltered electron transfer between $(BChl)_2$, BPh and Q_I, but are devoid of Q_{II} which is lost during the preparative procedures.

Addition of ubiquinone-10 to the RC–phospholipid–octane solution (fig.2B) has two effects. It increases the amount of $(BChl)_2$ seen to be stably oxidized on a ms timescale following a flash. The enhancement indicates that the added ubiquinone reconstitutes 30–50% of the RCs that evidently also lost their Q_I during preparative procedures. Figure 2B also shows that the addition of extra ubiquinone slows the re-reduction of $(BChl)_2^+$, in the back reaction, to a halftime of 165 ms. This slowing suggests that the added ubiquinone also reconstitutes the Q_I to Q_{II} electron transfer reaction (step 5 of scheme 1). In aqueous-detergent media the return of an electron to $(BChl)_2^+$ from Q_{II}^- is substantially slower than from Q_I^- although the observed kinetics are variable [32]. Support for the reconstitution of the Q_I to Q_{II} electron transfer reaction in the RC–phospholipid–octane solution is presented in fig.2C which shows the effect of *o*-phenanthroline, an inhibitor of the Q_I to Q_{II} electron transfer reaction ([31] and see [1]). In the presence of *o*-phenanthroline the $(BChl)_2^+$ reduction halftime reverts to that shown in fig.2A for RCs without Q_{II}.

3.3. *Steady state currents in planar RC-phospholipid membranes*

Figure 3A shows that steady state illumination of the RC-phospholipid membrane does not generate net electric currents. However, the addition of ferrocytochrome *c* (ferricytochrome *c* is ineffective) to one side of the membrane promotes a light-induced current as shown in fig.3B. The direction, but not the magnitude of the light-induced current response is dependent upon the side of the membrane to which ferrocytochrome *c* is added.

The addition of ubiquinone-10 to the RC–phospholipid–octane solution enhances the light-induced current responses as shown in fig.3C. The ubiquinone-induced enhancement of the current response probably arises from the reconstitution of Q_I in those RCs that lost their Q_I during the preparative procedure and from the reconstitution of Q_I to Q_{II} electron transfer and possibly even subsequent transfer to the excess ubiquinone 'pool' (Q_p) in the membrane. This conclusion is supported by the dramatic effect of

o-phenanthroline shown in fig.3D, which in inhibiting the Q_I to Q_{II} reaction reverts the light-induced current response to a level approaching that shown in fig.3B.

The photocurrent responses show a transient peak current which relaxes in the light to a steady state level. A transient discharging current in the opposite direction to the peak current is observed when the light is extinguished.

3.4. *The action spectrum of the transient peak current across planar RC-phospholipid membranes*

An action spectrum of the peak current response (●) generated during steady state illumination of planar membranes containing RCs and supplemented with ubiquinone-10 is shown in fig.1. The reasonable correspondence of the action spectrum to the absorption spectrum of the RC demonstrates that the electrical current observed arises from light absorbed by the RC.

3.5. *Flash-induced currents in planar RC ubiquinone phospholipid membranes*

Figure 4 presents further investigations using a series of single turnover xenon flashes spaced 25 ms apart to activate the RC–ubiquinone membranes. Figure 4A shows, in agreement with the steady state illumination results of fig.3, that no net electrical current is detected following any of the flashes unless ferrocytochrome *c* is added to one side of the membrane (fig.4B). The measured current integral (typically 4×10^{-10} A . s . cm^{-2}) matches the number of reducing equivalents available as estimated from the RC concentration of the RC–phospholipid–octane solution. No differences in the transient current kinetics were observed when laser pulses (pulse duration ~15 ns) were used to excite the RC–phospholipid membranes.

Two important points are revealed in fig.4 that are pertinent to considerations dealt with in section 4 regarding the disposition of the RCs in the membrane. In fig.4B the first flash yields only a small response while subsequent flashes elicit much larger current transients. However, fig.4C shows that the diminished response following the first flash can be overcome by addition of potassium ferricyanide to the side of the membrane opposite to the ferrocytochrome *c*.

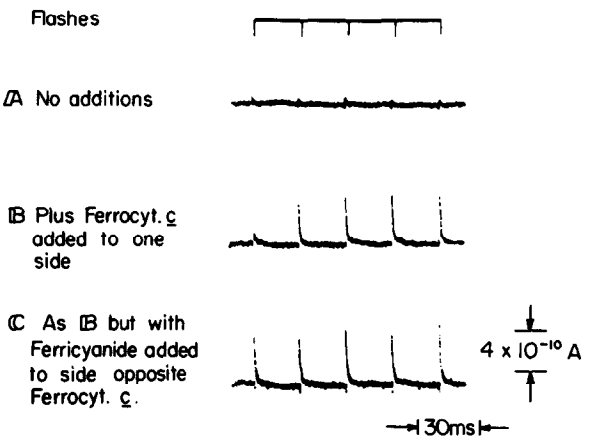

Fig.4. Flash-induced electrical currents across RC–ubiquinone–phospholipid membranes. The RC in the RC– phospholipid–octane solution was 8.3 μM. Ubiquinone-10 was added at 180 μM to this solution before membrane formation. (A) RC–ubiquinone–membrane alone. (B) As (A) but with 25 μM ferrocytochrome *c* added to one side of the membrane. (C) As (B) but ~20 mM potassium ferricyanide added to the side of the membrane opposite to that containing ferrocytochrome *c*. Because of the time constant of the voltage–clamp circuit, the duration of the recorded flash-induced currents is 1–2 orders of magnitude larger than both the flash duration, and the sum of the electron transfer rates from ferrocytochrome *c* to the ubiquinone.

4. Discussion

The following conclusions and interpretations emerge from this report.

(1) RCs can be solubilized in phospholipid octane. The flash-induced electron transfer reactions of the RC in this solution are improved if supplemented with ubiquinone-10 to reconstitute the partial loss of Q_I and total loss of Q_{II}. These findings complement the reports in [20,21].

(2) Black lipid bilayer membranes can be formed from the RC (and ubiquinone)–phospholipid–octane solution. These membranes support RC-driven light-induced electric currents directed across the membrane.

(3) The experiments of fig.3,4 lead to the view that the RCs, generally considered to be functionally vectorial [4–13] and structurally asymmetric [33,34] are deposited in the membrane as an equal mix of directionally opposing populations.

General support for this model comes from the observations that:

(i) Currents are not detected in RC membranes alone;

(ii) The direction, but not the magnitude of the current response is dependent on the side of the

membrane to which the ferrocytochrome *c* is added.

Further evidence for two opposing RC populations can be inferred from the flash excitation results where only a small current transient is detected following the first turnover, but not following subsequent turnovers. On the first turnover both sets of RC populations are activated, but their opposing current transients effectively cancel. However, by the second turnover, delivered 25 ms later, only the RC population accessible to ferrocytochrome *c* is returned to a functional state, while the other population is still essentially in the photochemically inactive $(BChl)_2^+$ BPh Q_I Q_{II}^- (or Q_p^-) state. The addition of the membrane-impermeant potassium ferricyanide to the side opposite to the side containing ferrocytochrome *c* chemically oxidizes only the RC population inaccessible to the ferrocytochrome *c*, thereby enabling a net current transient following the first turnover.

(4) There are indications that electron transfer is possible on the RC–ubiquinone membranes through Q_I and Q_{II} to at least part of Q_p. Single turnover flashes of the kind shown in fig.4 elicit current transients up to at least 10 turnovers before diminishing. Indeed, the peak current integral response to steady state illumination is typically 10-fold larger than the current integral obtained by single turnover flash activation. The occurrence of electron transfer between Q_I and Q_{II} is also supported by the attenuation of *o*-phenanthroline of the light-induced steady state currents.

(5) The possible sources of the steady-state currents (fig.3C) remain unclear. One possibility is that Q_p cycles the reducing equivalents back as hydrogens to the ferricytochrome *c*, in effect translocating H^+ across the membrane in an electrically-silent manner (see [35]). Alternatively, it is possible that the Q_p^- formed in the light reacts with molecular oxygen in the medium; this possibility is supported by the finding that steady-state currents, but not the transients, are eliminated under anaerobic conditions.

Acknowledgements

We thank Heather Bonner for excellent technical assistance and Peggi Mosley for the production of the manuscript. This work was supported by USPHS grant GM 12202 to P.M. and PHS grant GM 27309 and NSF grant 09042 to P.L.D.

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