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Photoelectric currents across planar bilayer membranes containing bacterial reaction centers: the response under conditions of multiple reaction-center turnovers

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The characteristics of the photocurrent response activated by continuous illumination of planar bilayer membranes containing bacterial reaction centers have been resolved by voltage clamp methods. The photocurrent response to a long light pulse consists of an initial spike arising from the fast, quasi-synchronous electron transfer from the reaction center bacteriochlorophyll dimer, $BChl_2$, to the primary quinone Q_A. This is followed by a slow relaxation of the current to that promoted by secondary, asynchronous multiple electron transfers from the reduced cytochrome *c* through the reaction centers to the ubiquinone-10 pool. Currents derived from cytochrome *c* oxidation that occurs when cytochrome *c* is associated with the reaction center or when limited by diffusional interaction from solution are recognized. Changes of the ionic strength and pH in the aqueous phase, and the clamped membrane potential (± 150 mV), affect the electron-transfer rate between cytochrome *c* and $BChl_2$. In contrast, the primary light-induced charge separation between $BChl_2$ and Q_A, or electron transfer between Q_A on the ubiquinone pool are unaffected. During illumination of reaction center membranes supplemented with cytochrome *c* and a ubiquinone pool, there is a small but significant steady-state current which is considered to be caused by the re-oxidation of photoreduced quinone by molecular oxygen. In the dark, after illumination of reaction centers supplemented with cytochrome *c* and a ubiquinone pool, there is a small amount of reverse current resulting from the movement of charges back across the membrane. This reverse current is observed maximally after 400 ms illumination while prolonged illumination diminishes the effect. The source of this current is uncertain, but it is considered to be due to the flux of anionic semiquinone within the membrane profile; this may also be the species that interacts with oxygen giving rise to the steady-state current. It is postulated that when the reaction centers are contained in an alkane-containing phospholipid membrane, in contrast to the in vivo situation, the semiquinone anion formed in the Q_B site is not tightly bound to the site and can, by exchange-diffusion with the membrane-quinone pool, move away from the site and accumulate in the membrane. However, in the absence, more quantitative work superoxide anion, resulting from O_2 interaction with semiquinone of Q_A, Q_B or pool cannot be excluded.

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Abbreviations: $BChl_2$, bacteriochlorophyll dimer; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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Introduction

The reaction center in photosynthetic bacteria catalyzes the initial reactions of light-induced electron transfer. Absorption of a photon by the reaction centers of the bacterium *Rhodobacter sphaeroides* results in the transfer of an electron

from a bacteriochlorophyll dimer, $BChl_2$, via bacteriopheophytin (and possibly a monomeric BChl) to a ubiquinone-10 molecule bound in the Q_A site to form $BChl_2^+ Q_A^-$ (for recent reviews, see Refs. 1–3). The $BChl_2^+$ is then reduced by a *c*-type cytochrome. The Q_A^- in turn reduces another ubiquinone associated with the reaction center Q_B site, to form Q_B^-. The Q_B^- is retained in the site until light activates the formation of Q_A^- again which reduces Q_B^- to the doubly reduced quinol; after protonation this is considered to be released into the quinone pool as the neutral quinol (QH_2).

The reaction membrane center of *Rb. sphaeroides* spans the membrane [4–6] with the cytochrome *c* and Q_B located on or near to opposite sides of the reaction center and the membrane. Thus, light-induced electron transfer catalyzed by the reaction center generates transmembrane electric currents and potentials.

The transmembrane electrogenic nature of these reactions have been recognized from in vivo studies on natural membranes for some time, reflected by the electrochromic responses of the carotenoid molecules in the membrane [7,8], by membrane-potential-dependent shifts in redox equilibria between cytochrome *c* and the $BChl_2$ [9], and by enhanced fluorescence response [10]. Such light-induced events have more recently been measured directly as electric currents and potentials across reaction centers incorporated into planar phospholipid membranes [11–18], in monolayers on solid support [14,19–21] and on the interfacial region of immiscible liquids [22]. Tiede [23] has recently reviewed many aspects of these approaches.

In earlier reports [13–15] we described the photoelectric response observed under conditions in which the $BChl_2$ complement of the reaction centers was restricted to a single electron turnover. We found that electron transfer from the photoexcited $BChl_2$ to Q_A and from ferrocytochrome *c* to the light-generated $BChl_2^+$ are both electrogenic and occur in series across the membrane. In contrast, we found that electron transfer from Q_A^- to Q_B contributes little or nothing to the photoelectric response. These results were entirely consistent with conclusions made from the above-quoted work on native membrane. They have also received considerable support from more recent electrical studies which, in addition, have provided evidence that suggests that a small electrogenic reaction may occurin concert with formation of the quinol in the Q_B site upon the transfer of an electron from Q_A^- to Q_B^-; this was interpreted as due to the response of protons moving into the dielectric to the Q_B site to form the neutral quinol [16,18].

Introductory studies that applied steady state rather than flash illumination to planar reaction center bilayer membranes [12] focused on the reaction center catalyzed transmembrane transport of charge from one aqueous phase containing ferrocytochrome *c* to the other containing ubiquinone-*o*. Events more closely associated with the reaction center itself were largely obscured. In this report we describe results from steady-state activation that is on a diminished time scale of inquiry than the earlier steady-state study [12] and is a logical extension of our single turnover flash studies [13–18]. In this work we have examined by assay of transmembrane currents and voltages induced by brief steady state illumination the interactions between the membrane-associated reaction center and cytochrome *c* that is added to one aqueous phase, and between the reaction center and ubiquinone contained within the membrane bilayer.

Materials and Methods

Preparative methods

Preparation of phospholipids. Bovine brain phosphatidylserine and egg phosphatidylcholine (PC) were prepared following the procedures in Refs. 24 and 25, respectively. Egg phosphatidylethanolamine (PE) was obtained from Supelco, Bellefonte, PA. All the phospholipids were stored in chloroform at $-80\,^\circ$C until use.

Preparation of reaction centers and their transfer to an alkane solution. Reaction centers were prepared from *Rb. sphaeorides* R26 cells following the method of Clayton and Wang [26] and of Feher and Okamura [27]. The reaction centers were stored in a 10 mM Tris-HCl (pH 8.0), 0.1% lauryldimethylamine *N*-oxide (LDAO) solution at $-80\,^\circ$C until further use. For membrane formation, the reaction centers were transferred from

the detergent solution to an octane-phospholipid solution following the previously described procedure [15]. Reaction centers (0.3–0.5 mg protein equivalent) were added to a presonicated phospholipid dispersion (10 mg total PC/PE/phosphatidylserine 3:3:1, w/w). Following further sonication for 15 min, $CaCl_2$ (final concentration, 100 mM) and 2 ml octane were added and the mixture vortex-stirred for 4 min. The mixture was subsequently centrifuged to yield an optically clear upper octane phase containing reaction centers and phospholipids. Excess octane was removed by evaporation to yield a solution containing reaction centers at approx. 10–15 μM. Addition of ubiquinone-10 (Sigma, St. Louis, MO) to the 'membrane-forming' solution, followed by brief sonication (5 s), was found to reconstitute the ubiquinone-10 lost from the Q_A site and to restore partially (approx. 70%) the Q_B complements as well as to provide a ubiquinone pool within the membrane [13–15].

Formation of planar bilayer membranes containing bacterial reaction centers. The membranes were formed by blowing an aliquot of the octane-reaction center solution across a 1 mm diameter hole punched in a thinned wall section of a Teflon beaker in a Perspex outer container [28]. The lipid films thinned to form uniform bilayer reaction center membranes that were black to reflected light.

Electrical measurements were recorded using two nonpolarizable silver-silver chloride electrodes, shielded from the light, and connected to a voltage clamp for recording the current at constant potentials. The clamp output was displayed on a storage oscilloscope (Tektronix 7613, Tektronix Inc., Beaverton, OR), or on a Digital oscilloscope (Nicolet Explorer IIIA, Nicolet Instrument Corp., Madison, WI) interfaced to a PDP 11/10 computer system capable of storing and averaging data. When black, the reaction center-membranes had a resistance of 10^7–10^8 $\Omega \cdot cm^{-2}$, a capacitance of 0.5–0.6 $\mu F \cdot cm^{-2}$, and contained approx. 10^9 reaction centers per cm^2.

Light activation was from a mercury arc lamp (30 W) filtered through 1 cm of water, and directed onto the reaction center-membrane with a light guide. The end of the light guide was placed very close to the membrane to avoid illumination of the torus. The diameter of the light guide was chosen to optimize the light-induced signals without significant illumination of the torus. Compared to the one used, we found that smaller diameter light guides chosen to illuminate only a fraction of the center of the bilayer yielded qualitatively the same results although of course the light-induced signals were smaller. The duration of the light pulses was controlled by a fast (0.5 ms time constant) shutter (Uniblitz 225L), linked to a pulse generator. It was calculated from current measurements as a function of light tensity, that the average number of photons absorbed by each reaction center was typically 100 s^{-1}. In the majority of measurements illumination was between 100–500 ms. Stirring of the aqueous phases had no detectable effects on the light-induced signals.

Strategies for the detection of light-induced currents across planar reaction-center membranes

The contribution of the first turnover and secondary turnovers, made possible by interaction with cytochrome *c* and ubiquinone-10, were separated experimentally by making use of the geometry of the reaction center-membrane as described in Fig. 1.

Formation of reaction center-membranes from an alkane solution of reaction centers and phospholipid results [13,15] in a distribution throughout the membrane of two equal reaction center populations positioned in the membrane with opposite orientation (Fig. 1A). Under these conditions, light activation does not evoke an electrical response because the opposing currents generated by the light-driven electron transport in each reaction center population cancel.

In contast to the above situation, a photocurrent response is recorded when ferrocytochrome *c* is added to one aqueous phase allowing multiple turnovers in one of the two reaction center populations (Fig. 1B). In this situation, the oppositely aligned reaction center population remains restricted to a single turnover. Since the initial turnover of electron transfer from $BChl_2$ to Q_A in the two populations are vectorially opposing, their currents cancel; thus, the recorded current is due to the initial fast reduction of the light-generated $BChl_2^-$ by the adsorbed ferrocytochrome *c* on the

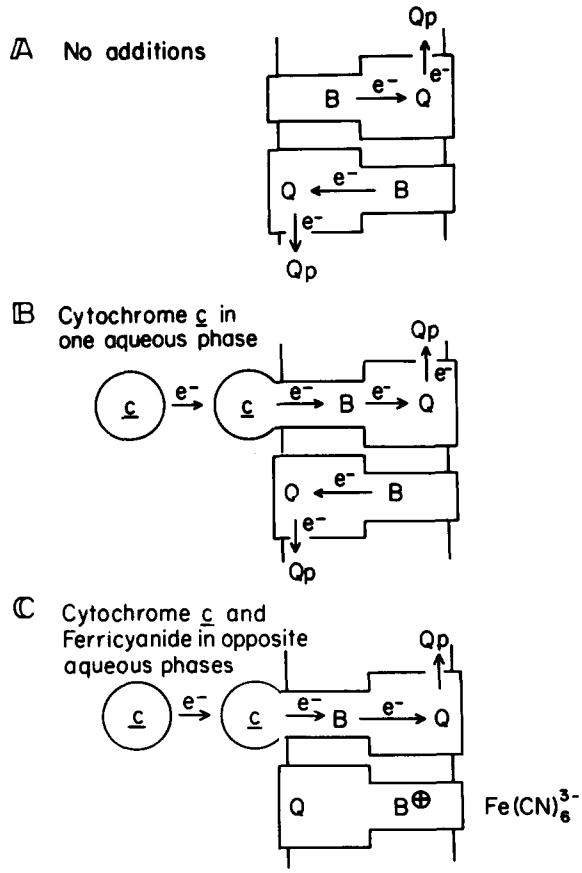

Fig. 1. Experimental conditions used to obtain the secondary turnover current, and the initial turnover current. (A) As formed, the membrane contains two equal reaction-center populations, but with their $BChl_2$ complement accessible to opposite aqueous phases. Without added cytochrome *c*, both reaction center populations are restricted to a single turnover. However, their vectorially opposing electron-transfer reactions cancel, and no light-induced current is detected. (B) Addition of reduced cytochrome *c* to one aqueous phase supports secondary turnovers in one reaction center population. The oppositely aligned reaction center population remains restricted to a single turnover. Thus, the current from the first turnover, $BChl_2$-to-Q_A electron transfers, cancel out; currents recorded thereafter will arise from cytochrome *c* oxidation and electron transfer through the reaction center. (C) Addition of potassium ferricyanide to the aqueous phase opposite to the cytochrome *c* causes the oxidation of the $BChl_2$ complement inaccessible to the cytochrome *c*. The light-induced current therefore contain contributions from both the initial turnover and secondary turnover responses of the one reaction-center population.

first turnover [15] and then to the slower, subsequent secondary turnovers as solution ferrocytochromes exchange with bound ferricytochrome *c* on the one reaction center population (Fig. 1B).

In order to observe the initial synchronous turnover of one reaction center population, potassium ferricyanide was added to one aqueous phase in order to oxidize the $BChl_2$ complement close to that side. The redox potential established after addition (typically, about +520 mV) is at equilibrium sufficient to cause the chemical oxidation (more than 90%) of the $BChl_2$ complement accessible to the ferricyanide. The actual extent of oxidation could not be determined directly; however, from the promptness in the response of light-induced current after ferricyanide addition, there is good reason to consider that equilibrium was approached and that this reaction center population was essentially inactivated. The oppositely aligned reaction center population is unaffected by the addition of ferricyanide and with no further addition it can be studied alone restricted to one turnover even with steady illumination. Addition of ferrocytochrome *c* to the other aqueous phase and of ubiquinone to the reaction center-membrane permits multiple turnovers as required [13–15].

The use of *o*-phenanthroline, which is known to inhibit electron transfer from Q_A to Q_B by displacing the ubiquinone molecule from the Q_B site [29–31], restricts the system to one turnover even in the presence of cytochrome *c* and a quinone pool. The effectiveness of *o*-phenanthroline in the present experiments relies on the finding that it is slow to cross the bilayer membrane so it can be used to inhibit one of the reaction center populations from transferring reduction equivalents into the quinone pool; if its effects are examined within minutes of addition a clear response is obtained [15]. However, another effect of *o*-phenanthroline that we have noted (see later) is its ability to displace ubiquinone from the Q_A site in these hexane-phospholipid-reaction center films; this also takes place on the minutes time-scale.

Finally, in some experiments, ascorbate (100 μM) was added to the aqueous phases (a) to ensure that the reaction center $BChl_2$ complement was completely reduced prior to activation (some oxidation of $BChl_2$ can occur when reaction centers are in the bilayers); and (b) to provide a sink of reducing equivalents for the cytochrome *c*. It should be mentioned that ascorbate, added to

one side of a reaction center membrane (ferricyanide on the other), did not itself promote multiple turnovers of any significance to the experiments.

Results

Characterization of the current response to pulsed illumination

Fig. 2A shows the photocurrent response obtained under conditions of scheme C of Fig. 1 (i.e., where one reaction center population is inactivated with ferricyanide prior to light excitation). Onset of illumination evokes a current response with results from the initial synchronous turnover and subsequent asynchronous turnovers of the active reaction center population accessible

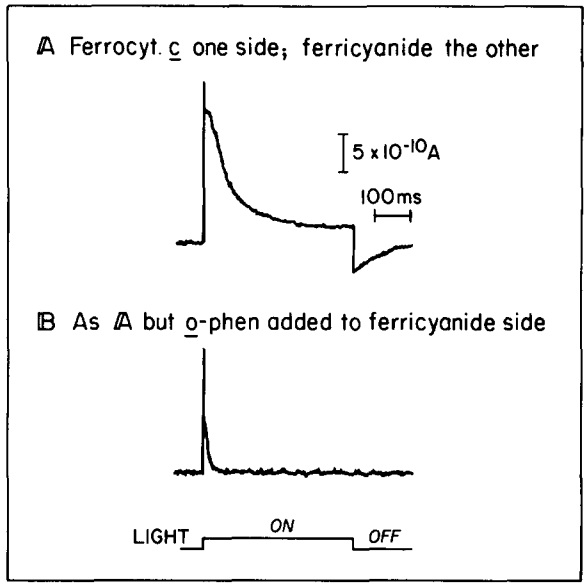

Fig. 2. The light-induced current response: effect of *o*-phenanthroline. Membrane was formed from an octane phospholipid reaction center (8 μM) solution supplemented at the time of formation of the solution with 100 μM ubiquinone-10. Both aqueous phases contained 0.01 M NaCl (pH 6.0). (A) Reduced cytochrome *c* (25 μM) and potassium ferricyanide (1 mM) were added to opposite aqueous phases; conditions as described in Fig. 1C. (B) Current response due to a single turnover. *o*-Phenanthroline (1 mM) from a 500 mM ethanolic solution added to the ferricyanide-containing aqueous phase. An equivalent amount of ethanol solvent added had no effect on the current response (not shown).

to both the cytochrome *c* and the ubiquinone pools. The photocurrent response of Fig. 2A consists of a transient peak, followed by a slow relaxation to a steady-state level. After the light is turned off a reverse current is detected. More detailed descriptions of these current responses are presented in the following sections.

The initial turnover current

The initial turnover contribution to the photocurrent response can be separated experimentally from the manifold responses described in Fig. 2A. One way to do this, as outlined in the Materials and Methods section, is by the addition of *o*-phenanthroline to the aqueous phase containing ferricyanide to prevent electron transfer from Q_A^- to Q_B in the active population and thereby to restrict each reaction center to only one turnover and hence the oxidation of one cytochrome *c*. With *o*-phenanthroline present, the photocurrent response consists of a spike and only a brief slower phase which relaxes promptly to zero current (Fig. 2B); close inspection (not shown) shows the course of decay during illumination to be described reasonably well by two first-order kinetic phases. Hence, it is reasonable to conclude that the peak current has contributions from both electron transfer from $BChl_2$ to Q_A, and from the oxidation of only one adsorbed ferrocytochrome *c* to $BChl_2^+$ to yield a final state comprising ferricytochrome *c* Q_A^-, beyond which no further reaction is observed. Moreover, after illumination, since this state will relax only in the seconds time domain, no reverse current is recorded on the milliseconds time-scale shown.

The initial spike seen on top of the peak in Fig. 2A is expected to result almost entirely from the 30% of the reaction centers that do not contain a reconstituted Q_B molecule. Because this population supports one turnover it is expected to display only a short current response; this population will also contribute to the spike seen in Fig. 2B. If this identification is correct then the amplitude of the spike should be more sensitive to the light flux than slower secondary events; this is clearly shown to be the case in Fig. 3.

The secondary turnover current

The contribution from secondary turnovers to

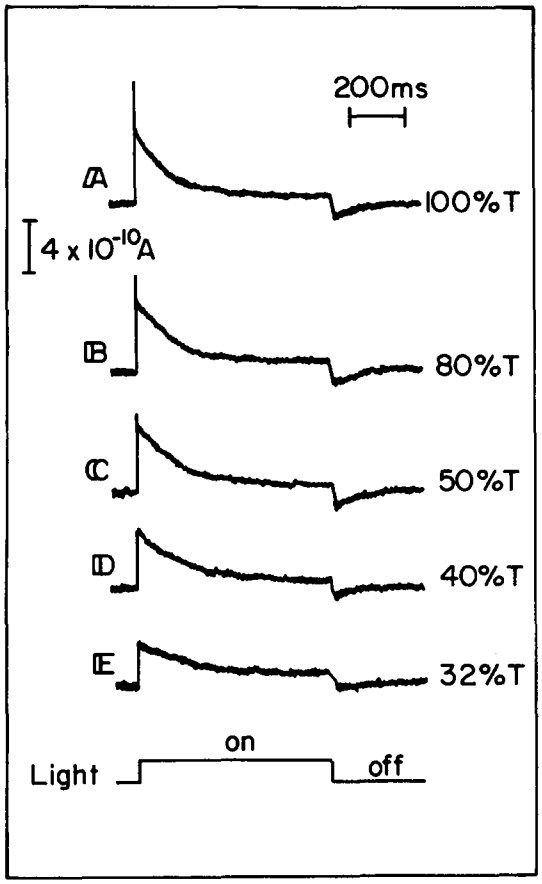

Fig. 3. The effect of the light intensity on the current response. Membranes formed from an octane-phospholipid reaction center (12 μM) supplemented with 100 μM ubiquinone-10. Both aqueous phases contain 0.01 M NaCl (pH 6.0). Reduced cytochrome *c* (5 μM) was added to one aqueous phase, whereas 1 mM ferricyanide was added to the opposite aqueous phase. Light intensity, maximal at 100 photons per reaction center per s, was attenuated with neutral density filters. Their transmission is indicated to the right of each record.

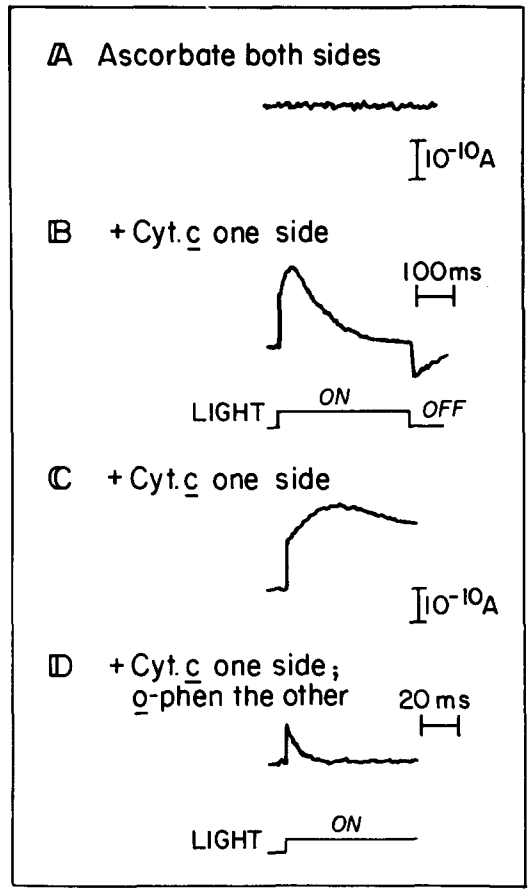

Fig. 4. The secondary turnover current response. Membranes were formed form an octane-phospholipid reaction centers (8 μM) solution supplemented with 100 μM ubiquinone-10. Both aqueous phases contained 0.01 M NaCl (pH 6.0), and 100 μM sodium ascorbate. The ascorbate was added to keep both reaction center populations reduced for maximal initial turnover, but is not essential. (A) No further additions. (B) The secondary turnover response induced by cytochrome *c* (Cyt. *c*) (25 μM) added to one aqueous phase. The time and current calibration are the same for the trace in A and B. (C) Conditions as described in B, except that the time-scale calibration is 20 ms. (D) Addition of *o*-phenanthroline (*o*-phen) (1 mM) to the aqueous phase opposite the cytochrome *c* side. Same current and time-scale calibration as in (C).

the photocurrent can be readily observed by comparing the single turnover response of Fig. 2B with the trace of Fig. 2A. Thus, in Fig. 2A, the extended lifetime of the peak current, the steady-state current and the reverse current are clearly all generated by the asynchronous secondary turnovers. From the difference in the integrals between the peak currents of Fig. 2A and B we calculate that individual reaction centers can undergo approx. ten turnovers during the experimental illumination period.

A more direct way of measuring the photocurrent response that registers only the secondary turnovers of one reaction center population is achieved when the oppositely aligned reaction center population is permitted to undergo only one turnover (see Scheme B of Fig. 1). Fig. 4B shows that under these conditions the initial spike is missing, consistent with its identification with

events occurring on the first turnover between $BChl_2$ and Q_A. The net photocurrent response consists of a braod peak current, a slow relaxation to a steady-state level and, when the light is switched off, a reverse current. Two rise phases are observed in the peak current response (Fig. 4C); a fast phase with a time constant limited by the shutter opening and a slower rise-phase with an approx. 10 ms time constant. The fast phase is interpreted as originating in the electrogenic electron transfer from the adsorbed cytochrome *c* to the light-oxidized $BChl_2$ on the first turnover. The absorbed state of cytochrome *c* required for this interpretation is expected at the prevailing cytochrome *c* concentration if the dissociation constants (values typically fall in the range 10^{-6}–10^{-5} M; Refs. 32–35) measured in detergent or in reconstituted phospholipid apply to this system. Some quantitative support for this assertion is presented in the next section. In contrast, the slow phase is interpreted as monitoring the electrogenic events accompanying secondary turnovers of electron transfer from pool cytochrome *c* through the reaction center to the quinone pool. Again, confirmation of this conclusion can be obtained by use of *o*-phenanthroline added to the side opposite to that containing cytochrome *c*. As shown in Fig. 4D, *o*-phenanthroline, expected to abolish all electron transfer except for the first turnover, indeed has a dramatic effect, leaving a current contribution characteristic only of electron transfer from the adsorbed ferrocytochrome *c* to the light-generated $BChl_2^+$ on the first turnover. Further, quantitative support for this identification is described below.

The effect of the ubiquinone and cytochrome c pool sizes on the photocurrent response

Fig. 5A shows the effect of varying the amount of added ubiquinone on the light-induced current response at a constant but non-limiting (see Fig. 5B) cytochrome *c* concentration. Fig. 5A demonstrates that the amplitudes of both the initial peak current and the secondary turnover current measured 20 ms following the onset of illumination increase with the amount of added ubiquinone. The immediate effect (0–15 Q per reaction center $(BChl)_2$) on the increase of the inital peak current and later, as discussed elsewhere [13–15], is due to the reconstitution of Q_A in that reaction center population which lost its ubiquinone during the extraction procedure. Beyond this a general trend of increased current is seen as the membrane ubiquinone pool size is enlarged.

Fig. 5B shows the effect of varying the amount of added cytochrome *c* on the light-induced current response, at constant but non-limiting ubiquinone-10 concentration. It is clear that the secondary turnover current response depends on the concentration of reduced cytochrome *c*. The increased amplitude of the initial peak current seen at cytochrome *c* concentrations above 10^{-6} M is in accord with the micromolar dissociation constants reported [32–35], and is most probably due to the adsorption of cytochrome *c* to the reaction center. Thus, in contrast to the slower, diffusion-limited interaction of pool cytochrome *c* with the reaction center, the adsorbed cytochrome *c* is capable of rapid electron transfer to $BChl_2^+$; in these measurements this phase is not time-resolved from the $BChl_2$ to Q_A electron transfer and hence contributes to the initial current peak.

The steady-state current

After the secondary turnover current has ceased, a steady-state current is observed (Fig. 2A). While only a fraction of the current encountered during the first ten or so turnovers, the steady-state current ($(2–5) \cdot 10^{-10}$ A) is significant and equivalent to about 3–5 turnovers per s. Fig. 2B, which shows that the steady-state process is inhibited by *o*-phenanthroline, supports the conclusion that multiple turnovers are a prerequisite for the steady-state current.

The reverse current in the dark after illumination

In Fig. 2A we noted that cessation of illumination evokes a reverse current of opposite polarity to the peak current. The reverse current subsequently relaxes to zero current with a time constant of approx. 140 ms. This response cannot be due to the simple back reaction of electron transfer from Q_A^- to $BChl_2^+$, since under these conditions the light generated $BChl_2^+$ is reduced by cytochrome *c* to prevent this reaction. The source of the reverse current most likely is associated with events linked to subsequent secondary turnovers of the reaction center that involve the

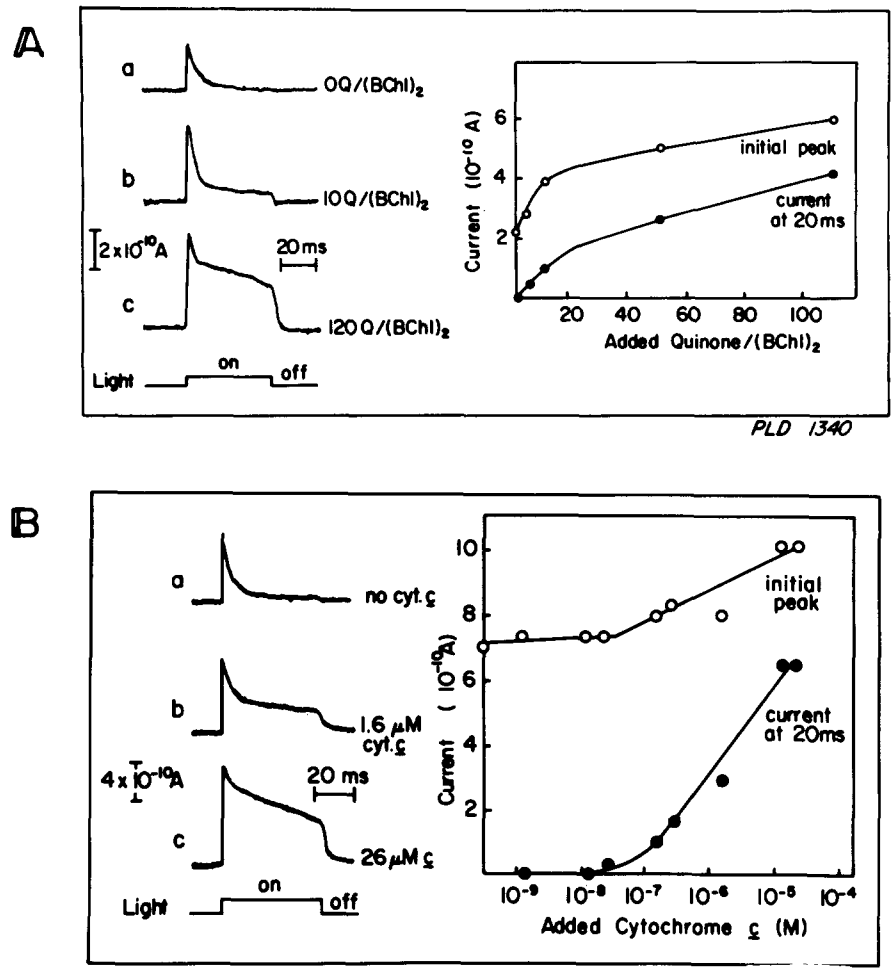

Fig. 5. The effect of added ubiquinone-10 (A) and added cytochrome *c* (B) on the light-induced current response. Membrane was formed from an octane-phospholipid-reaction center solution (7 μM). Both aqueous phases contained 0.01 NaCl (pH 6.0); one aqueous phase contained 1 mM potassium ferricyanide. Both the initial peak current, and the secondary turnover current measured after 20 ms were related to the quinone or cytochrome *c* pool sizes. (A) The effect of added ubiquinone-10: ubiquinone-10 was added directly as a solid at the time of formation tot he octane-phospholipid reaction center solution. Since the partition of the quinone between the bilayer and lipid torus is not determined, the actual bilayer quinone concentration is unknown; hence the variation in ubiquinone concentration is presented as a Q/reaction center ratio established by measuring the $BChl_2$ absorption in the membrane-forming solution. Approx. 50% of the reaction centers retain their bound Q_A during the extraction procedure [15]. Reduced cytochrome (10 μM) was added to one aqueous phase opposite to that containing ferricyanide. (B) The effect of added cytochrome *c*: varying amounts of reduced cytochrome *c* were added to the opposite to that containing potassium ferricyanide. The membrane forming solution was supplemented with 200 μM ubiquinone-10.

quinone pool. As expected for such a proposal, the calculated integral of the reverse current, although only a fraction of the forward current, multiplied by the Faraday, is several-fold larger than the reaction-center population. Further support for this is provided in Fig. 6 which shows that the amplitude of the reverse current is affected by the duration of the illumination pulse. With relatively short light pulses (less than 100 ms) no significant reverse current is detected. However, longer light pulses yield a reverse current which reaches a maximal amplitude after 400 ms illumination and decreases thereafter.

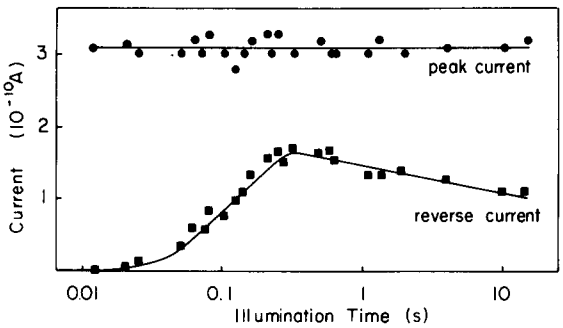

Fig. 6. The effect of the light duration on the reverse current transient. Membranes were formed from an octane-phospholipid reaction center (8 μm) solution supplemented with 100 μM ubiquinone-10. Both aqueous phases contained 0.01 M NaCl (pH 6.0). Potassium ferricyanide (1 mM) and reduced cytochrome *c* (25 μM) were added to opposite aqueous phases. The duration of the light pulse was varied from 10 ms to 10 s. The effect of the light duration of the amplitude of the reverse current and the initial peak current is shown.

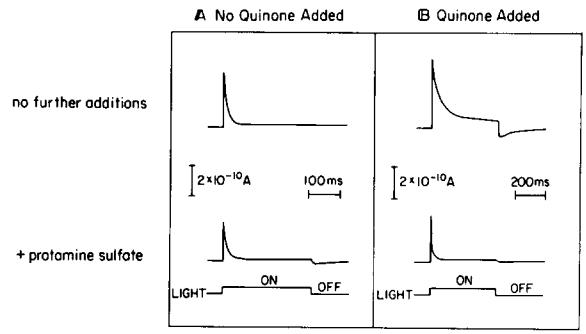

Fig. 7. The effect of protamine sulfate on the light-induced current response. (A) Membranes formed from octane-phospholipid reaction center (7 μM) solution. The aqueous phases (pH 6.0, 0.01 M NaCl) contained ferricyanide (1 mM) and cytochrome *c* (10 μM) on opposite sides. Protamine sulfate (25 μM) was added as indicated. (B) As (A), except ubiquinone (100 μM) was added to the membrane forming solution.

The photocurrent response to the contribution to electron transfer from the solution cytochrome c

Although the molecular exchange or electron-transfer reactions between the reduced cytochrome *c* pool in solution and the oxidized adsorbed cytochrome *c*, and from the reduced Q_B to the quinone pool do not themselves appear to contribute directly to the photocurrent response [15], the electron-transfer rates between the pools and reaction-center components will obviously affect the secondary turnover photocurrent response. In this section we show how changes in the steady-state photocurrent response result from an alteration of the rate of interaction between the solution cytochrome *c* pool and the reaction center in the membrane. This was achieved by: (a) altering the binding equilibrium between the reactants with changes in the ionic medium (see Ref. 32); and (b) altering the redox equilibrium by the application of transmembrane potentials (see Ref. 9). These effects are described below.

Effects of protamine sulphate

In Fig. 7 we show the effect on the photocurrent response of adding the polycation protamine sulphate to one aqueous phase under conditions in which the reaction center population is either restricted to one turnover or permitted to undergo multiple turnovers. The single turnover response of Fig. 7A was obtained with reaction center-membranes devoid of added ubiquinone, but containing cytochrome *c* on one side. With no protamine sulphate added, the photocurrent relaxes to zero and, as expected, after illumination no reverse current is detected. With protamine sulphate added to the aqueous phase containing cytochrome *c*, the photocurrent response is modified. There is an attenuation of the light-induced peak current, and after illumination, the appearence of a small transient reverse current which relaxes with a time constant of approx. 100 ms. This alteration in the current response is consistent with protamine sulphate effectively abolishing any significant ferrocytochrome *c* reduction of the light-generated $BChl_2^+$ and thereby, in the absence of added quinone and hence Q_B, restricting current generation to electron transfer between $BChl_2$ and Q_A. Thus, an observed reverse current is expected, caused by the back reaction from the reduced Q_A^- to $BChl_2^+$.

Fig. 7B shows the effect of protamine sulfate when excess ubiquinone is present (the conditions correspond to Scheme C of Fig. 1). Protamine sulphate under these conditions also has a dramatic inhibitory effect on the photocurrent response of multiple turnovers.

We envisage that the polycation adsorbs to the negatively charged reaction center membrane

causing the displacement of cytochrome *c* from the membrane and in particular from its reaction-center binding site.

Effect of ionic strength

The ionic strength dependence on the photocurrent response evoked by single turnover (no added cytochrome *c*) or secondary turnover conditions is shown in Fig. 8 (top left). There is no effect of ionic strength on the electron transfer between $BChl_2$ and the quinone acceptor pool. However, Fig. 8 (top right) shows that in the presence of cytochrome *c* the photocurrent response generated by secondary turnovers is attenuated both at high (greater than 10 mM NaCl) and low (less than 100 μM NaCl) salt conditions. Fig. 8 (bottom) shows these effects on a continuous basis of changing salt concentration. The results suggest that at high ionic strengths the effect of salt is similar to that of protamine sulfate, while at low ionic strengths electron transfer from the cytochrome *c* is restricted for other reasons. Similar effects at low ionic strength have been reported by Overfield and Wraight [32] working with reaction centers reconstituted in phospholipid vesicles; they attributed the phenomenon to an increased binding of the cytochrome *c* to the bilayer constituents, which leads to a diminished mobility and a rate-limiting exchange of cytochrome *c* molecules with the reaction center and solution.

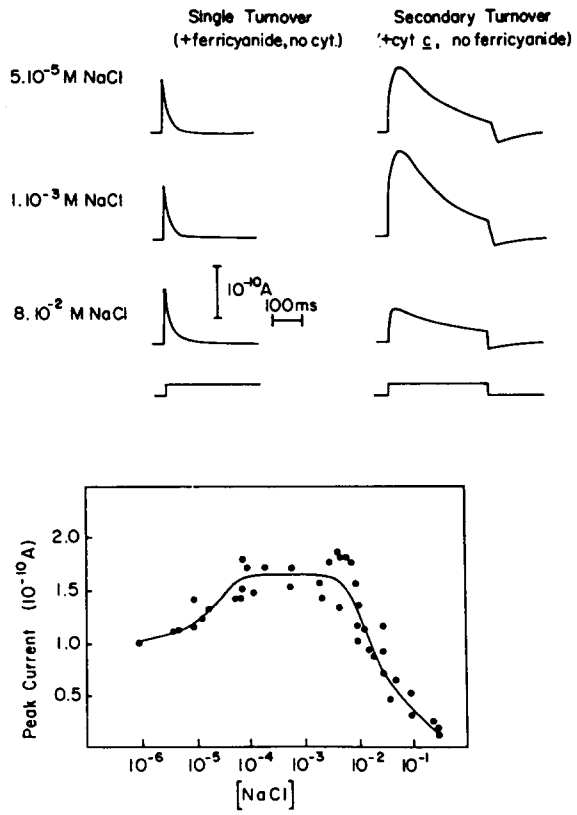

Fig. 8. Ionic strength dependence of the light-induced currents. Membranes formed from octane-phospholipid reaction center (7 μM) solution at pH 6.0. The single turnover responses obtained by adding 1 mM ferricyanide to one side. Secondary turnover responses were obtained by addition of cytochrome *c* (10 μM) to one side only and no ferricyanide but 100 μM sodium ascorbate. The relation between the secondary turnover peak and the salt concentration is shown in the plot.

Effect of pH

Fig. 9 shows that the photocurrent response of secondary turnovers of the reaction centers is not dependent on the pH of the aqueous phase between pH 5.5 and 9. However, both at low and high pH, the photocurrent response is strongly attenuated with midpoint values of 4.8 and 9.6. This profile is similar to the observed pH dependence of the oxidation rate of cytochrome *c* by reaction centers in proteoliposomes [32]. Again, the source of the effect may reside in the binding reactions. The isoelectric points of cytochrome *c* and reaction center are 6 and 10.5 (see Ref. 32) and although the molecular details of the interaction between these proteins are not yet known, the diminished photocurrent above pH 9 and below

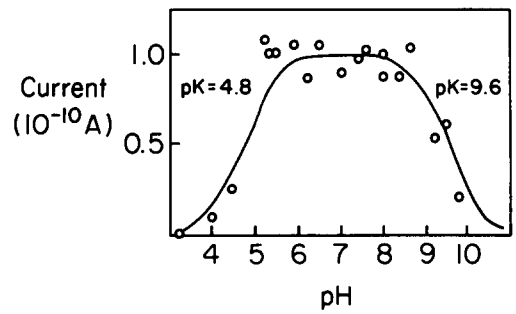

Fig. 9. The pH dependence of the secondary turnover current. Membranes formed from octane-phospholipid-reaction enter (8 μM) solution. The secondary turnover currents were obtained as in Fig. 5. The plot shows the amplitude of the response peak vs. the pH of the aqueousu phase (both sides) which contained 0.01 M NaCl.

pH 5 probably resides in the neutralization of residues important for binding.

Effect of applied potentials on the electron transfer between cytochrome c and $BChl_2$

In all previous measurements, the voltage across the reaction center-membrane was clamped to zero throughout the course of the experiment. In Fig. 10 we examined the effect of applied potentials (± 150 mV) on the photocurrent response. These measurements were made under the conditions described in Fig. 1B. The application of an electric field that is expected to promote the cytochrome *c* oxidation (i.e., cytochrome *c* side negative, Q_A/Q_B side positive) has no effect on the current peak. However, when the direction of the field is reversed the amplitude of the peak current is diminished; hence, an opposing field hinders the electron transfer from ferrocytochrome *c* to $BChl_2^+$, presumably by altering the apparent free-energy difference between the two in accordance with interpretations made in studies on chromatophores [9]. Extrapolation of the voltage clamp dependence yields a value of 350 mV (cytochrome *c* side positive; Q_A/Q_B side negative) that would have to be applied under these conditions to obtain zero current.

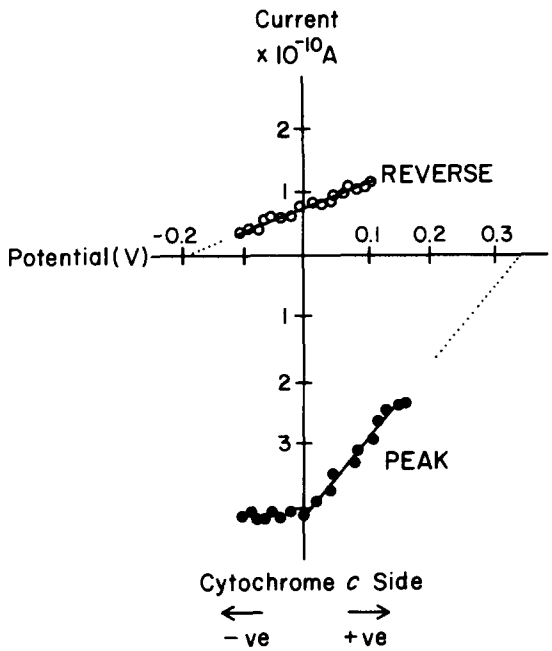

Fig. 10. The effect of applied potentials on the light-induced currents. Membranes formed from octane-phospholipid-reaction center (8 μM) solution. Conditions as described in Fig. 5, except that potassium ferricyanide was added. The amplitudes of the peak current at 20 ms at the maximal reverse current were measured at different applied potentials.

Parallel experiments (not shown) done on the effects of applied potentials on the currents seen upon $BChl_2^+Q_A^-$ formation and recombination (conditionsof ferricyanide on one side and no cytochrome *c* on the other) revealed little or no changes in either amplitude of the current signal assignable to $BChl_2^+Q_A^-$ formation or of the kinetics of charge recombination. It is worth noting, however, that potentials applied across reaction center monolayers, have been reported to effect both the yield of $BChl_2^+Q_A^-$ formation [21] and recombination kinetics [17,20,37]. However, the fields necessary to induce these relatively small effects were several-fold larger in magnitude; with the lower fields applied here to the bilayer membranes, such effects would not be readily detectable; a similar absence of electric field effects on the charge recombination from semi-ubiquinone to $BChl_2^+$ in reaction center bilayer membranes have been reported [17]. Thus, it can be concluded that the changes to the forward current, shown in Fig. 10, reside in the cytochrome *c* to $BChl_2$ electron-transfer step alone.

Effect of applied fields on the reverse current

Fig. 10 also shows the amplitude of the reverse current that was discussed earlier in the subsection The Reverse Current in the Dark after Illumination (p. 76). The current response displays an opposite field dependency to that of the cytochrome *c* to $BChl_2^+$ reaction and, in further contrast, is linear over the range examined. Extrapolation of the voltage clamp dependency of the current indicates that a value of 180 mV (cytochrome *c* side negative) must be applied to obtain a zero reverse current.

Discussion

Current observed in the light

We have applied simple experimental strategies to study the cytochrome *c*-reaction center-

ubiquinone-10 system in planar membrane bilayers. The utility of this system seems clear. Despite the fact that there is no natural vectorial asymmetry induced in the reaction center population upon forming the membrane bilayers, this problem can readily be overcome by appropriate additions of redox agents, inhibitors or natural acceptors and donors to one or another of the aqueous phases.

Another characteristic of the reaction-center membrane is that the highest reaction center concentration is in the order of one hundredth of that in the natural membrane, which yields electrical signals that are accordingly smaller [15,17]. Efforts to increase the reaction center concentration beyond the level used in these experiments have not been successful so far.

We have shown that the cytochrome *c*-reaction center interactions and electron transfer in these planar membranes to be of a type recognized in vivo [43] and in vitro [32–35]; they are readily reconstitutable and will provide a valuable complimentary approach to spectroscopic studies of the interaction of cytochrome *c* with the reaction center and membrane. In the case of reconstitutions of the ubiquinone-10 pool and its interaction with the Q_B site, only approx. 70% of the Q_B complement can be reconstituted by ubiquinone-10 added to the membrane forming solution. Thus far in our experiments (see also Ref. 15) there is commonly about a 30% reaction center complement whose reactions go only as far as Q_A; the reason for this apparently incomplete reconstitution of Q_B is uncertain.

The capacity of the ubiquinone-10 pool is severely restricted by the volume of the membrane interior; compared to the ferrocytochrome *c* available for the aqueous phase, this is minute. In view of this and in the absence of any intermediary redox reagent able to catalyze the transfer of reducing equivalents neutrally from the reduced quinone of the pool back to the oxidized cytochrome *c* (as in a simple chemiosmotic loop formulation [38]), it was anticipated that the photocurrent response would relax to zero current. However, a small but significant steady-state current is reported and we have done some exploratory work (unpublished data) to identify its origin. Thus far we have considered oxygen as a possible source of the effect acting to reoxidize the reduced ubiquinone back to the quinone form and so promote further reaction center turnover. Evidence to support this possibility comes from the finding that illumination of detergent-solubilized reaction center preparations supplemented with both ubiquinone-10 and cytochrome *c* does in fact result in oxygen consumption. Typical rates of 0.5–0.55 nmol O_2/s per nmol $BChl_2$ have been measured from reaction center suspension (1.3 μM) containing added ubiquinone-10 (200 μM) and cytochrome *c* (250 μM). Consistent with this phenomenon being the source of the steady-state current, we have also shown that illumination of reaction-center membranes under lowered O_2 tension induces lower steady-state currents; however, no detailed quantitation has been done. It is worth noting, however, that quinols such as ubiquinol are well known to be unreactive with molecular oxygen (see Ref. 39). The quinone species that reacts with oxygen may be the more reactive semiquinone form [39,40] either in the Q_B site and/or, as suggested in the next section, free in the membrane.

Currents observed in the post-illumination period

The source of the reverse current decaying with an approx. 140 ms half-time is, as in the case of the steady-state current, not certain; however, the sources of the two currents may be related. The reverse current seems very unlikely to be due to the simple reversal of electron transfer through the reaction center from the reduced Q pool to oxidized cytochrome *c*. Since under the prevailing ionic conditions (10–20 mM NaCl) any ferricytochrome *c* formed by the light-activated reaction center will be competed off the reaction center by the excess reduced form and will also be reduced by ascorbate. We can exclude the source being an electrochemical proton gradient, involving H^+ moving from the cytochrome *c* side to the Q_A/Q_B side, because the phenomenon is unaffected by the presence of buffer (it is worth noting that the number of H^+ involved would be minuscule, namely in the order of 10^8) and also because the electrical gradient is clamped at zero potential. We can also discount the possibility of the current arising from the transfer of a charged species from one bulk phase to another; in this regard, the

known candidates are Na^+ or Cl^-; buffer and ascorbate can be discounted, since the phenomenon is independent of their concentration. Also, we have not favored the possibility that the source of the current is some form of a reduced oxygen species, such as the superoxide anion radical formed during the interaction of oxygen with the reduced quinone. This conclusion is based on the rate of oxygen uptake by isolated reaction centers being too slow to accumulate a product that could give rise to the reverse current. Moreover, our preliminary measurements made in the presence of lowered oxygen tension while diminishing the steady-state current, did not diminish the reverse current. However, without detailed quantitation this possibility cannot be ruled out (see Ref. 44).

Rather than O_2^-, another proposal that accounts for the steady state and the reverse currents is that the quinone pool, upon reduction, remains charged, for example, in the semiquinone anion (Q^-) form. In this proposal the reverse current will not be the result of any vectorial redox reaction, but will occur as the negatively charged semiquinone diffuses down its chemical gradient away from its site of generation. Since the Q_B site is closer to one side of the membrane, one component of this gradient will be directed vectorially across the membrane towards the cytochrome *c* side to yield a current like that observed, opposite in sign to that seen during the light-driven electron flow from the cytochrome *c* side to the Q_B side of the membrane.

Thus, the illumination time-dependence of the appearance of the reverse current (Fig. 6) will be a consequence of the quinone pool reduction. At longer illumination times a contribution to its attenuation could be due to its rate of reduction slowing with the increase state of reduction of the pool and approaching that of the diffusion. However, under prolonged illumination we have found that the reverse current only very slowly approaches zero; this is accounted for by the previous suggestion applied to the steady state current, that oxygen is slowly reoxidizing the semiquinone (see Ref. 39) from both sides of the membrane to reform quinone, and hence to permit some regeneration of Q^- at the Q_B site thereby tending to reform the gradient.

If the discussions have any validity and a semiquinone anion is the species responsible for the reverse current, then several criteria must be satisfied. Thus, it would be expected that quinone from the pool once reduced to the Q^- state at the Q_B site can diffuse away from this site. This contrasts sharply with the situation native membrane in which the semiquinone is held firmly in the site, being released into the pool to any significance only after reception of a second electron (and the binding of two protons) to produce the neutral quinol. In the planar membrane system used in the present studies conaining not only phospholipid but also an uncertain amount of octane, the binding affinities of the quinones with binding sites, including Q_A, are clearly orders of magnitude weaker than in the native system; as we have discussed, during the preparation not only Q_B, but a large fraction of Q_A partitions into the solvent. We note also in other work with reaction centers in aqueous media that certain water-soluble benzoquinones reduced to the semiquinone in the Q_A site appear to diffuse away into solution (Gunner, M.R. and Dutton, P.L., unpublished results); although the conditions and properties are different in the two systems there are clear parallels.

Another requirement would be that protonation of Q^- is not favored, since this would eliminate the reverse current. p*K* values of benzosemiquinones have been reported to be in the range of 4–5 in aqueous media, and one unit higher in aqueous-propanol-acetone. A measurement made on ubisemiquinone indicates a p*K* of 5.9 in aqueous-propanol (7 M)-acetone (1 M) and an estimate of 6.45 has been reported in methanol (see Ref. 40). Hence, even with an aqueous phase at pH 6.0 as used in our experiments, it is reasonable to consider that the semiquinone anion could be a significant part of the steady-state reduced quinone population supported in this system.

The effect of applied electric fields on the current responses; concluding structural and functional comments

The contribution of electron transfer between cytochrome *c* and the $BChl_2$ to the separation of charge across the membrane has been seen before as an electrochromic bandshift of the carotenoid

complements of the native membrane [8,9] or as a voltage measured directly in a system identical or similar to the one used in the present paper [13–16]. In the native membrane the redox equilibrium between cytochrome *c* and $BChl_2$ has been demonstrated to shift in concert with the transmembrane potential. All these approaches indicate that the electron-transfer reaction takes place across approaching one half of the transmembrane dielectric distance. The work is in agreement with these earlier different measurements. Linear extrapolation of the photocurrent attenuation curve to zero current (Fig. 10) yields an e.m.f. for this electron-transfer reaction of 350 mV. Dark equilibrium oxidation-reduction (redox potential titrations indicate that the difference between the two midpoint potentials of horse heart cytochrome *c* in Tris-HCl [41] and the $BChl_2$ in the reaction center [42] (both involving one electron and no net proton exchange) is approx. 185 mV. Thus, while it is appreciated on a theoretical basis that the photocurrent-applied potential relationship is certainly more complicated than a simple linear relationship, and on a practical level the dielectric profile of this membrane is not homogeneous, the result obtained is consistent with earlier studies that suggest the electron transfer in the reaction from cytochrome *c* to light-oxidized $BChl_2$ spans approaching approx. Half of the dielectric distance of the reaction center-reconstituted membrane.

It is of considerable interest in view of the substantial membrane dielectric span indicated by these several electrical measurements that the X-ray crystal structure suggests (Tiede, D.M., personal communication) that although the distance from the magnesiums of the $BChl_2$ are estimated to be approx. 2 nm away from the iron of the cytochrome *c* heme, the $BChl_2$ magnesiums are placed approx. only 1 nm into the membrane profile from the aqueous edge; it seems unlikely that the effective dielectric constant between $BChl_2$ and the cytochrome *c* would be lower than the rest of the membrane protein profile due to its close proximity to the phospholipid head groups, charged amino acids and water. This intriguing discrepancy warrants further work.

The other electric-field-sensitive current was the post-illumination reverse current that the results suggested is due to the transmembrane diffusion of an ion rather than electron transfer. As discussed above the species responsible for this would be a semiquinone anion moving across the profile of the membrane. As such the e.m.f. of -180 mV for the reverse current expresses the value of the transmembrane chemical potential difference (an activity gradient across the profile of the bilayer) of anionic semiquinones generated in the light. This latter observation draws attention to a form of free energy conserved by photosynthetic membrane protein systems that has received little or no recognition. Most studies have been aimed at describing the conversion of light energy in terms of a redoxpotential difference (ΔE_h) between cytochrome *c* and quinone, and of an electric potential ($\Delta\psi$) expressed across the membrane from one aqueous phase to another. In this case we are seeing, in addition, the free energy expressed as a chemical potential gradient generated within the membrane. While there are reasons to believe (see below) that in vivo the semiquinone anion may not constitute a free member of the pool, the above phenomenon provides an important demonstration: it is conceivable that analogous gradients of quinol or quinone may be generated across the membrane of the native system during steady-state activity. Unfortunately, these will be difficult to demonstrate; however, before trying, the O_2^- possibility should be ruled out.

In the intact photosynthetic membranes the ΔE_h between the reduced quinone together with the oxidized cytochrome *c* is used to drive another membrane redox protein, the quinol-cytochrome *c* oxide reductase that generates further transmembrane electrical potential and effects proton transport which provides the driving force for ion movements and ATP formation. This is, of course, a major distinction between the native and the artificial system described here. However, apart from the absence of the quinol-cytochrome *c* oxidoreductase, it seems clear in the native membrane that the quinone reduced on the Q_B site of the reaction center is not permitted to leave the Q_B site as the semiquinone. It is worth noting that if the semiquinone did leave as the anion and became free within the native membrane, as we have considered it may do in our artificial alkane phospholipid membrane, then it would diffuse

under the additional influence of $\Delta\Psi$ and tend to collapse the electrochemical gradient of protons. Moreover, the presence of free semiquinone may, as we have mentioned, enhance the generation of the potentially damaging superoxide. The role of the Q_B site in vivo clearly is designed to avoid these possibilities.

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