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SPECTROPHOTOMETRIC AND VOLTAGE CLAMP CHARACTERIZATION OF MONOLAYERS OF BACTERIAL PHOTOSYNTHETIC REACTION CENTERS

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Bacterial photosynthetic reaction centers from *Rhodopseudomonas sphaeroides* have been spread on an air/aqueous interface, compressed, and transferred quantitatively to either glass or transparent, tin oxide-coated slides. These assemblies permit the concomitant measurement of both optical and electrical activities to be made on protein films under voltage-clamp conditions. Optical spectra of the monolayer-coated slides reveal characteristic reaction center absorptions. Linear dichroism spectra of the monolayers indicate that the reaction center is aligned on the air/aqueous interface with an angle of inclination which is essentially the same as it is with respect to the membrane plane in vivo. The kinetics of the light-induced absorbance changes of the reaction center in the deposited films are essentially unaltered from those in solution; however, there is some loss in the extent of photochemical activity. Measurement of the light-induced electrical transients shows capacitative charging and discharging currents, which can be readily associated with the reaction center bacteriochlorophyll dimer to ubiquinone electron transfer. The extent of the photochemical activity detected by the voltage-clamp is at best only 10–12% of that measured by optical assay. This suggests that on the air/aqueous interface, the reaction centers must be predominately oriented with opposing directions of electron transfer, having only a slight, variable tendency to align with the ubiquinone directed toward the aqueous phase. In spite of the present shortcomings, these assemblies appear to be uniquely useful to study the effect of clamped potentials on the kinetics and mechanisms of electron transfer.

Introduction

The successful incorporation of isolated bacterial photosynthetic reaction centers into planar bilayers [1–3] separating aqueous phases has provided a useful experimental approach with which to examine by voltage-clamp techniques the correlation between electron-transfer reactions and electrical, charge-separating events directed across the membrane. The usefulness of this work is enhanced by the fact that electron-transfer reactions occurring within the reaction center have been quite well characterized using spectrometric methods down to 10 ps, and have been described in recent reviews [4–6]. Briefly, the completion of the forward light-intitiated electron transfer within the reaction center complex occurs between the bacteriochlorophyll dimer, $(BChl)_2$, and a ubiquinone, Q. This transfer of an electron between $(BChl)_2$ and Q occurs in at least two steps. First, an electron transfer between $(BChl)_2$ and a monomeric bacteriopheophytin, BPh, occurs within 10 ps and possibly involves an intervening monomeric BChl [7]. The final transfer from BPh to Q

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Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin.

occurs with a 100–200 ps halftime. In the absence of a secondary electron donor to the oxidized $(BChl)_2$ (i.e., $(BChl)_2^{+}$) or a secondary acceptor for the reduced Q (i.e., $Q^{\bar{\cdot}}$), an electron returns to the $(BChl)_2^{+}$ from $Q^{\bar{\cdot}}$ with a 60–80 ms halftime at room temperature.

In the planar bilayer-containing reaction centers, the charge separation between $(BChl)_2$ and Q has been shown [3] to charge approximately half the membrane capacitance, suggesting that this represents a charge separation extending over about 20–25 Å measured vectorially normal to the plane of the supporting membrane. The voltage measured following a single turnover flash in such a system is typically 1–2 mV. This relatively small value is consistent with the low density of reaction centers in the membrane, typically close to 10^9 reaction centers/cm^2 which compares with the approx. 10^{11} reaction centers/cm^2 in the natural membranes [8]. Technical problems have prohibited the incorporation of higher reaction center densities in the planar bilayer. Further inconvenience arises from the fact that the reaction centers deposit in two equal, vectorially opposed populations so it is necessary to disable one of the populations prior to experimentation [1–3]. Finally, the low reaction center density together with the small area (less than 1 mm^2) of the planar bilayer makes spectrometric characterization of the reaction centers in the bilayer difficult.

In an alternative approach to the study of the coupling between electron transfer and electrical potential generation, we have initiated studies to overcome the problems attendant with the planar reaction center phospholipid bilayers. We are developing methods to form closely packed, vectorially uniform reaction center monolayers deposited onto glass and transparent electrode surfaces from an air/aqueous interface. In this paper, we describe both optical and electrical properties of what appear to approximate to single reaction center monolayers and multilayer assemblies. The strong near-infrared absorption of the reaction center BChl and BPh chromophores (electronic transitions) provides a means to monitor the functional activity and angular orientation of the reaction center in monolayer films. Placement of the monolayer films between electrodes permits the net, vectorial charge separation perpendicular to the electrodes to be measured by voltage-clamp techniques and provides a means to assay the degree to which the reaction centers are aligned along a single direction in the reaction center films. Taken together, these measurements suggest a structure for the monolayer film and demonstrate the feasibility of using the monolayer assemblies to make concomitant measurement of optical and electrical activities of monolayer films under voltage-clamp conditions.

Materials and Methods

Reaction center preparation

Reaction centers of *Rhodopseudomonas sphaeroides* R-26 were prepared as described previously [9]. The reaction centers were suspended in 0.05% lauryldimethylamine *N*-oxide, 10 mM Tris-HCl (pH 8.0) at a concentration of 50 μM, and with a protein purity indicated by a 280 nm/800 nm absorbance ratio of 1.3.

Formation and deposition of reaction center coatings

As described by Blodgett and Langmuir [10] (see also Refs. 11 and 12 for reviews), a compressed monolayer of aliphatic molecules can be transferred quantitatively to a solid support by raising a hydrophilic support up through the monolayer from the aqueous phase, leaving the support with the more hydrophilic side of the layer in contact with the support and the more hydrophobic side exposed to the air. Alternatively, the transfer can be done by lowering a support with a hydrophobic surface through the monolayer from the air, depositing a layer with the more hydrophobic side against the solid support. Sequential passes through a series of monolayers can lead to a specifically designed multilayer array (see, however, Refs. 13 and 14 for evidence on the likelihood of significant reorganization of deposited mono- and multilayers).

Reaction centers were introduced onto the air/aqueous surface by dripping the detergent solution down a glass rod. The aqueous phase was a solution of 2 mM phosphate buffer, 1 mM $MgCl_2$ (pH 6.7) and the temperature 25°C. The reaction centers were detected on the surface by a depression of the surface tension as measured by an electronic linear motion transducer [11]. Increasing the density of reaction centers on the surface by decreasing the surface area produces a decrease in the surface tension. A saturation of the surface was often found to occur at a surface pressure near 30 dyne/cm (a surface tension of about 40 dyne/cm), beyond which further compression of the surface layer yielded only small changes in the surface tension. At this compression, the reaction center density on the surface corresponded to $4.5 \cdot 10^{12}$ reaction centers/cm^2. However, the existence of the transition in the surface pressure vs. area curves at 30 dyne/cm was found to vary with different reaction center preparations, and in these cases the surface tension changed continuously as the film was compressed in this region. Variation in the surface pressure vs. area isotherms could possibly arise from variations in the amount of tightly bound phospholipid or detergent in the reaction center preparations [15–17] and these details will be examined in a future study.

The reaction centers were deposited onto glass or tin oxide slides while maintaining the surface pressure at 30 dyne/cm^2. This compression was chosen, since the resulting density of $4.5 \cdot 10^{12}$ reaction centers/cm^2 is very close to the density predicted for a single monolayer of the approx. 50 Å diameter [18,19] reaction center molecules, packed in a hexagonal array.

The reaction centers were deposited onto the glass or tin oxide in two ways. First, by raising a 2.5 cm square slide from beneath the surface at a rate of 1 cm/min. The slides were previously coated with a bilayer of arachidate, leaving the slides with a hydrophilic surface. When multiple reaction center coatings were built up, after the first reaction center coating had been deposited, a monolayer of arachidate was deposited on top of the air-dried reaction center coating by lowering the slide through the fatty acid monolayer. With the slide below the surface, the trough was cleaned and reaction centers again added to the surface, compressed to 30 dyne/cm surface pressure and deposited by raising the slide. In this procedure, each reaction center coating is deposited with the portion of the reaction center facing the aqueous phase (the more hydrophilic side) always directed towards the support.

An alternative approach was to deposit the more hydrophobic side of the reaction center (i.e., that facing the air) against the support by lowering a slide with a hydrophobic surface (prepared with a single arachidate deposition) held parallel to the air/aqueous interface until it just touched the surface.

The addition of 20 arachidate molecules per reaction center to the detergent-solubilized reaction center solution improved somewhat the uniformity and completeness of the transfer of the reaction centers from the aqueous interface to the solid support when preparing the multilayer samples. The presence of the arachidate in the reaction center monolayer altered neither the optical properties nor the electrical responses of single reaction center coatings, and this addition of arachidate was used routinely in the formation of the reaction center films described here.

In addition, in the case of the electrical measurements, four arachidate monolayers were deposited on top of the single and multiple reaction center coatings in order to provide a spacer between the reaction center layer and the second, mercury electrode (see below). This was found to be necessary, since interactions between the reaction centers and the evaporated metal or mercury electrodes were seen when these were placed directly on top of the reaction center layer (see Discussion).

Electrical measurements

The light-induced electrical transients were measured with reaction centers deposited onto tin oxide-coated slides, which provided a transparent electrode. The slide was held horizontally, and a second electrode was provided by a drop of mercury placed directly on top of the deposited layers. The tin oxide and mercury electrodes were connected to a voltage clamp and the light-induced, reaction center current transients were measured with the electrodes held at constant (zero) potential difference, and the light-induced potential generation measured with a high-impedance amplifier. The slides were illuminated from below with a focussed 8 V, 40 W lamp, filtered through 2 cm of water and a Corning 2418 red filter.

Spectrophotometry

Optical absorption spectra of the reaction centers deposited onto glass slides were recorded on a Johnson Foundation scanning double-beam apparatus. The spectra shown are the average of four, 4-min scans. Kinetics of the flash-induced $(BChl)_2$ oxidation and reduction were measured at 870 nm with the transparent tin oxide slides tilted at 45° to both the actinic light and the measuring beam.

Results

Optical properties and activities of reaction center monolayers

The optical properties of the reaction center coatings were examined in order to determine the number, activity, and with linear dichroic techniques, the angular inclination of the reaction centers with respect to the solid support.

The optical absorptions of the BPh and BChl near-infrared absorption bands in a single reaction center coating deposited onto both sides of a glass slide are shown in Fig. 1. The strong optical absorbtion of the monomeric BChl ($E_{800nm} = 288 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$) and BPh ($E_{760nm} = 125 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$) [9] can be recognized in the upper spectrum recorded with the slides in air. However, the absorption of $(BChl)_2$ at 865 nm is much smaller than expected; the spectrum resembles that in solution when the reaction centers are illuminated or have been treated with an oxidant to oxidize $(BChl)_2$ to $(BChl)_2^{+}$. The extent of the loss of absorption at 865 nm was found to be variable and preparation dependent; it would appear that in the reaction center coatings $(BChl)_2$ is susceptible to auto-oxidation.

To test this, a reaction center-coated slide was submerged into an aqueous solution of sodium ascorbate (100 μM) and a redox mediator, 2,3,5,6-tetramethylphenylenediamine (100 μM). Under these conditions, the reaction centers are not seen to dissociate from the slide and $(BChl)_2$ is found to become completely reduced. The second spectrum of the reaction center coating shown in Fig. 1 resembles very closely that of intact reaction centers. The absorbance of a slide with a single reaction center coating on each side is 0.0044 *A* at 800 nm; using an extinction coefficient of $2.88 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ this corresponds to a density on the slide of about $4.6 \cdot 10^{12}$ reaction centers/cm². This density is essentially the same as that calculated for the reaction centers compressed to a surface pressure of 30 dyne/cm on the air/aqueous interface, and indicates that the reaction centers are transferred essentially quantitatively as a monolayer onto the slide from the aqueous surface.

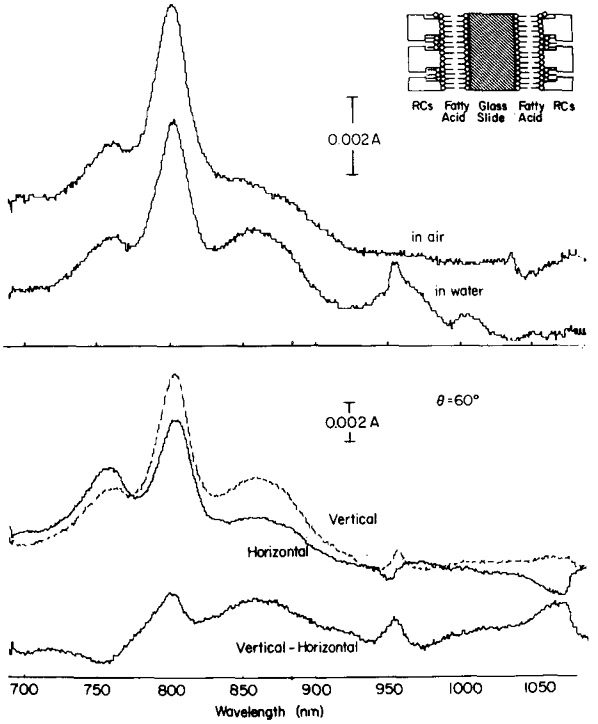

Fig. 1. Optical spectra of single reaction center monolayers. The fatty acid (arachidate) and reaction center (RC) monolayers were deposited onto a 2.5 cm square microscope slide as described in Materials and Methods. The inset shows a diagram of the cross-section of the slide. The top spectrum was recorded with the slide held in air. Immediately below is the spectrum of the same slide submerged in water, containing the reductant 2,3,5,6-tetramethylphenylenediamine (100 μM). With the slide in water, the lower spectra were recorded with either horizontally (parallel to the plance of incidence) or vertically (perpendicular to the plane of incidence) polarized light. The spectra were recorded with an angle of incidence of 60° and by using a calcite, prism polarizer.

The orientation of the reaction centers in the coating was examined by linear dichroism. The spectra taken with the reaction center coating submerged in water under reducing conditions are shown in the lower portion of Fig. 1. These spectra show that the 800 and 865 nm transitions absorb preferentially with light polarized parallel to the

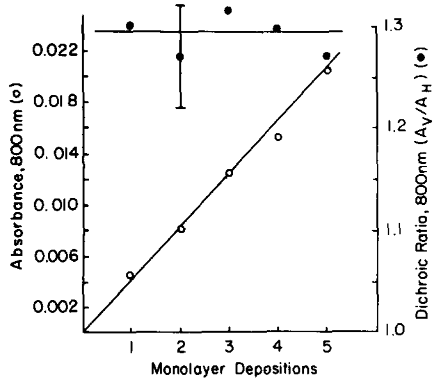

Fig. 2. Optical properties of reaction center multilayers deposited on glass slides. Each point represents a different slide which had various numbers of reactions center films deposited. The abscissa refers to the number of reaction center films deposited while raising the slide from beneath the air/aqueous interface as described in Materials and Methods. The left and right ordinates refer to the absorbance at 800 nm (both sides of the slide are coated) and the dichroic ratio (A_V/A_H) at 800 nm (angle of incidence, 60°), respectively. The spectra were recorded with the slide submerged in water under the reducing conditions as described in the text.

plane of the slide (vertical), having dichroism ratios, A_V/A_H, of 1.2 ± 0.05 and 1.6 ± 0.1, respectively; while the BPh 760 nm absorbance occurs preferentially with light polarized parallel to the plane of incidence (horizontal) with a dichroic ratio of 0.8 ± 0.05. These dichroic spectra are both qualitatively and quantitatively analogous to those of the reaction center in the oriented natural chromatophore membrane [20] and in oriented membranes of reaction centers reconstituted into phosphatidylcholine vesicles (Ref. 19 and Tiede, D. and Dutton, P.L., unpublished results). These results suggest that the reaction centers on the air/aqueous interface tend to assume an orientation with respect to the surface which is essentially identical to its orientation with respect to the membrane plane.

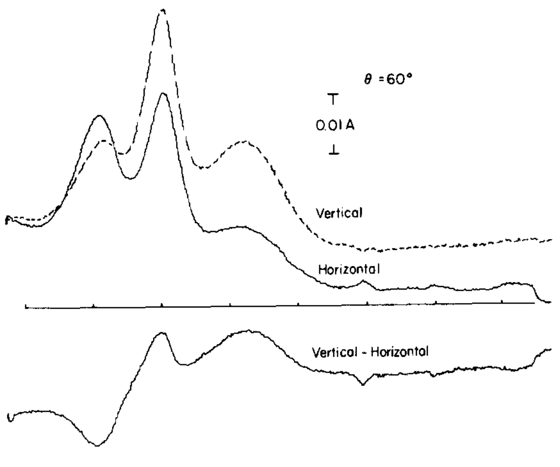

Fig. 3. Linear dichroic spectra of a slide coated (both sides) with five reaction center films in water. Conditions and preparations were as described in Fig. 2 and Materials and Methods.

Evidence for the ability to construct a multilayer array is shown in Fig. 2. Each point represents one of a series of slides that were coated with a different number of reaction center depositions. As described in Materials and Methods, each layer was deposited by raising the slide from beneath the air/aqueous interface. The slides exhibit a linear increase in the absorbance at 800 nm consonant with the number of reaction center coatings. This shows that each reaction center layer on the air/aqueous interface is transferred with a similar, high efficiency to the slide. In addition, assays of the linear dichroism spectra show that there is no loss in reaction center orientation with increasing reaction center depositions, as demonstrated by the consistency of the dichroic ratios measured at 800 nm which are also plotted in Fig. 2. The linear dichroism spectrum for the five-reaction center coating array is shown in Fig. 3. These data suggest that the monolayer technique can be used to construct a well ordered, multilayer array.

The dichroism spectra show that the reaction center coatings do not become disoriented by submersion in water for at least up to 1 h. These spectra are qualitatively similar to those obtained with the reaction center coatings in air, although in air the spectra are distorted by additional interference and reflection effects arising from the increased difference in the refractive index between the air and the coating [11]. Although $(BChl)_2$ was seen to be at least partially oxidized in the single reaction center coatings, $(BChl)_2$ was found to be less susceptible to auto-oxidation in the multiple-coating arrays.

These spectra demonstrate a close correlation between the structure of the reaction center in the coating with that in natural and reconstituted membranes. In addition, the photochemical activity of the coatings was assayed by measuring the extent and kinetics of the light-induced $(\mathrm{BChl})_2$ oxidation and its dark rereduction by $Q_I^{\cdot-}$. This was done by monitoring the flash-induced absorption changes at 870 nm. The $(\mathrm{BChl})_2$ oxidation and rereduction kinetics are shown in Fig. 4 for a four-reaction center coating a air, and these are compared with the kinetics for the native reaction centers in solution. $(\mathrm{BChl})_2$ was oxidized by a series of four xenon flashes spaced 40 ms apart, generating on this time scale of detection, the $(\mathrm{BChl})_2^{\cdot+}Q^{\cdot-}$ state. It can be seen that greater than 90% saturation of the photochemical reaction occurs with the xenon flashes used, and that the millisecond kinetics of the return of the electron from $Q_I^{\cdot-}$ to $(\mathrm{BChl})_2^{\cdot+}$ is unaltered from that observed for reaction centers in solution.

While the kinetics of the functioning reaction centers in the reaction center films appear to be unaltered from those in the native reaction center, the extent of $(\mathrm{BChl})_2$ oxidation appears to be reduced. For example, in the multilayer sample shown in Fig. 4 the absorption at 870 nm was measured to be 0.0075 A (reaction centers deposited on both sides of the slide), while the light-induced bleaching was found to reach only 0.0035 A. Because the optical spectrum of the BChls and BPhs in the reaction center coatings appear unaltered from that in the isolated reaction center, it would seem that the partial attenuation of the functional activity could arise, for example, from alterations in the quinone binding in the reaction center coatings.

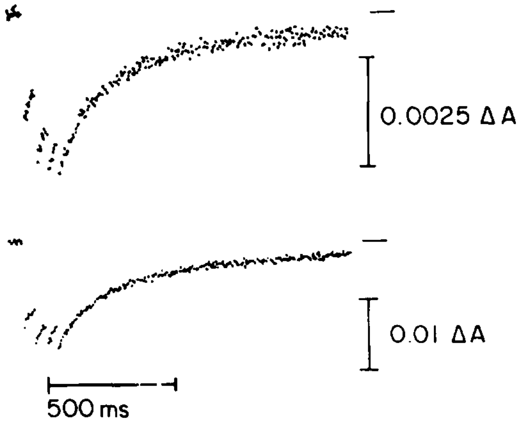

Fig. 4. Flash-induced optical absorption changes measured at 870 nm. The top trace shows the absorbance changes for a slide coated with four reaction center films deposited on both sides of the slide. The trace is the average of 16 recordings. Both the light-induced kinetic and the optical absorptions at 870 nm ($A = 0.0075$) were measured with the slide tilted at an angle of 45° with the measuring beam. The lower trace shows a similar light-induced kinetic recording for reaction centers suspended in 0.1% lauryldimethylamine *N*-oxide, 10 mM Tris-HCl (pH 8.0). The four xenon flashes were spaced 40 ms apart.

Light-induced electrical responses in reaction center coatings

The linear dichroism measurements have established that reaction centers are oriented with the same angle of inclination on the air/aqueous interface as they are with respect to the membrane surface in the natural membrane. However, these measurements cannot distinguish between a symmetric reaction center coating, in which the reaction centers are distributed between two equal and vectorially opposing populations, and an asymmetric layer in which the oriented reaction centers are all pointing in the same direction. Instead, the degree of symmetry was determined by measuring the net movement of charge across the reaction center coatings by voltage-clamp techniques. As described in Materials and Methods, the electrode pair was provided by deposition of the reaction center coating onto a tin oxide slide and a second, mercury electrode placed directly on top of the reaction center and arachidate coatings.

Fig. 5 shows the steady-state light-induced current transients measured for single reaction center coatings. The lower trace was obtained from a coating deposited with the portion of the reaction center layer facing the aqueous phase deposited against the tin oxide slide (the tin oxide slide was raised from beneath the air/aqueous interface). The light-induced current transients have been identified as arising from a simple capacitative charging by the reaction center photochemical reactions [1–3,22]. The initial spike arises from events occurring during the charge separation that leads to $(\mathrm{BChl})_2^{\cdot+}Q^{\cdot-}$ formation, and the width is dependent upon the intensity of the activating light. The current goes to zero as the steady-state $(\mathrm{BChl})_2^{+}Q^{-}$ charge separation is maintained and there is no net

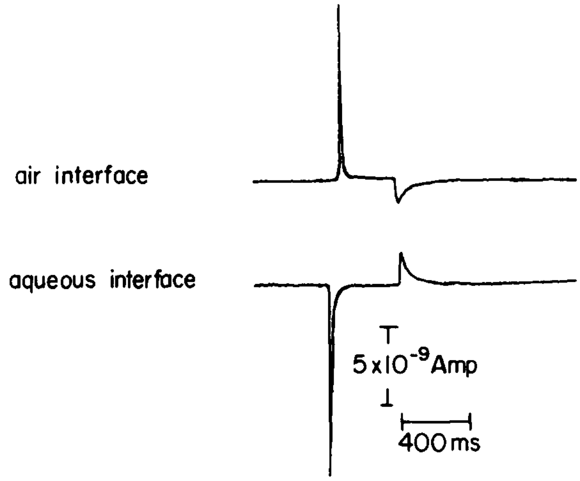

Fig. 5. Reaction center monolayer light-induced current transients measured with potential clamped to zero. The top trace was the response elicited from a slide in which the portion of the reaction center film facing the air on an air/aqueous interface was deposited against the tin oxide slide; the lower trace was recorded with a slide in which the portion of the reaction center film facing the aqueous solution on an air/aqueous interface was deposited against the tin oxide slide. The reaction center film in both cases was separted from the tin oxide and mercury electrodes by four arachidate monolayers. The 3 mm diameter mercury drop was the ground electrode.

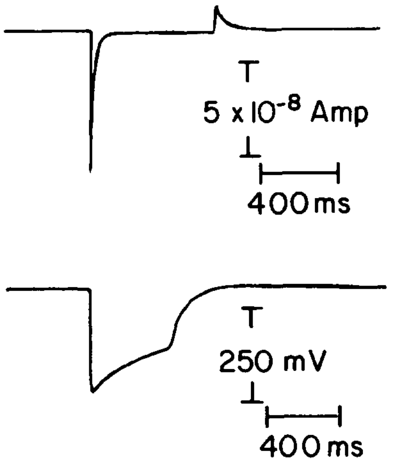

Fig. 6. Light-induced electrical transients for a five reaction center multilayer array. Reaction center films were deposited with the portion facing the aqueous phase deposited against the tin oxide slide. The top trace shows the light-induced current transient, measured with a potential clamped to zero; the lower trace shows the light-induced potential generation under zero current conditions. The mercury drop (3 mm diameter) was the ground electrode. Other conditions as in Fig. 5 in the text.

electron transfer between the reaction center coatings and the electrode. Finally, a discharging current is seen, rate limited by the 80 ms halftime of the back reaction Q_1^- to $(BChl)_2^+$, when the light is switched off. This result demonstrates that there is a net functional alignment in the direction of electron transfer within the reaction centers in the coating. This point is further emphasized in the top trace in Fig. 5, where the reaction center coating was deposited with the opposite orientation; here, the portion of the reaction center layer facing the air was deposited against the tin oxide slide (reaction centers deposited on the slide from above the air/aqueous interface). In this coating the same light-induced electrical transients are seen, except that the capacitative charging is in the opposite direction. These results not only suggest that there is a net alignment of the reaction centers in the coating, but also tend to rule out the possibility of artifacts in the light-induced electrical responses and suggest that the reaction centers tend to align vectorially on the air/aqueous interface with $(BChl)_2$ preferentially directed towards the air phase and Q nearer to the aqueous phase.

The light-induced current and potential transients for the five-coating multilayers are shown in Fig. 6. In this reaction center preparation the current integrals under the charging spike were found to reach $7 \cdot 10^{-9}$ C/cm^2 with potentials up to 300 mV; while in the single-layer samples current integrals were found to reach $2.3 \cdot 10^{-9}$ C/cm^2 with potentials measured up to 20 mV. Optical spectra show the $(BChl)_2$ to be approx. 3-fold more reduced in this multilayer sample than in the companion single-layer samples. This increase in the state of reduction of the $(BChl)_2$ and an electrical activity of the multilayers acting in series account for the increase in the current integrals and the proportionately larger increases in the potential generation. However, the extents of the electrical responses were seen to be extremely variable; for example, the potentials generated in the single-monolayer films varied from less than 1 to nearly 30 mV, depending upon the preparation and whether the film was soaked in reducing solutions. Intermediate values of about 5 mV were most common for untreated single coatings.

Comparisons of Electrical and Chemical Activities

The ability to measure both optical and electrical properties of reaction center monolayers on tin oxide slide provides the opportunity to make comparisons between the total photochemical activity measured optically with the net charge movement perpendicular to the electrode surfaces measured by the voltage clamp. This correspondence between the number of functioning reaction centers in the monolayer and the resultant electrogenic activity can be used to deduce information on the extent of the unidirectional alignment of the monolayer film.

Table I provides a tabulation of the electrical and optical data for a five-monolayer-coated slide. The back of the glass slide was cleaned so that the optical measurements reflected only those activities on the tin oxide surface. The column on the left lists the resistance and capacitance properties of the film measured in the dark, along with the light-induced capacitative charging integral, $4.1 \cdot 10^{-9}$ C/cm^2, and potential generation, 150 mV. The optical spectrum showed an absorbance at 800 nm of 0.0109 which corresponds to a reaction center density of about $22.7 \cdot 10^{12}$ reaction centers/cm^2. Since five monolayer deposition were made, this corresponds to the expected $4.5 \cdot 10^{12}$ reaction centers/cm^2 density for each monolayer. The light-induced chemical activity, however, only showed a ΔA of 0.001 at 860 nm which corresponds to a density of $5.2 \cdot 10^{12}$ reaction center/cm^2, indicating that only 23% of the reaction centers present on the slide are functioning. In an attempt to provide for a more complete reduction of the $(BChl)_2$ before illumination, the slide was soaked in a solution of 100 μM sodium ascorbate, 100 μM diaminodurene for 30 s, allowed to dry in air and then the electrical and optical activities were measured. The results are shown on the right column of Table I. After chemical reduction, the light-induced charging integral was found to increase to $6.8 \cdot 10^{-9}$ C/cm^2, with potential generation of 290 mV. The optical absorption at 800 nm was found not to change, however, the light-induced bleaching at 860 nm was found to increase to a ΔA of 0.00182, corresponding to $8.62 \cdot 20^{12}$ functioning reaction centers/per^2, which represent 38% of the total.

Since the oxidation of each $(BChl)_2$ within the reaction center can be associated with the displacement of one electron, this spectroscopic measurement of the number of functioning reaction centers provides a measurement of the number of charges moved, which can be compared directly to the charge movement or current integral detected by the voltage clamp. However, two factors must

TABLE I

COMPARISON BETWEEN THE OPTICAL AND ELECTRICAL ACTIVITIES OF A TIN OXIDE SLIDE COATED WITH FIVE REACTION CENTER (RC) DEPOSITIONS

Coating: tin oxide, four arachidate monolayers, five reaction center monolayer, four arachidate monolayers, mercury. Conditions for the electrical measurements were as described in Fig. 6, and the optical measurements were made as described in Figs. 3 and 4.

	Values measured in the dark	After chemical reduction
Resistance ($\Omega \cdot$cm^2)	$1 \cdot 10^8$	
Capacitance (F/cm^2)	$2 \cdot 10^{-8}$	
Peak current (A/cm^2)	$3 \cdot 10^{-7}$	$5.8 \cdot 10^{-7}$
Peak potential (mV)	150	290
Charging integral (C/cm^2)	$4.1 \cdot 10^{-9}$	$6.8 \cdot 10^{-9}$
A_{800}	0.0109 (corresponds to $22.7 \cdot 10^{12}$ reaction centers/cm^2)	0.0109
ΔA_{860}	0.0011 (corresponds to $5.2 \cdot 10^{12}$ reaction centers/cm^2, i.e., 23% of reaction centers present are working)	0.00182 (corresponds to 8.62 reaction centers/cm^2, i.e., 38% of reaction centers present are working)

be taken into account. Firstly, the five monolayers in the multilayer array act in series, so that the current integral for the multilayer capacitor will be expected to be proportional to just that of the individual reaction center capacitors; i.e., proportional to one-fifth of the total number of functioning reaction centers. Secondly, the electron transfer between $(BChl)_2$ and Q occurs across only a part of the individual, in series capacitors. For example, in reconstituted planar bilayer [3] and in the natural chromatophore [23,24], the $(BChl)_2$ to Q_I electron transfer is seen to occur across approx. one-half of the membrane capacitance, suggesting a net electron-transfer distance with respect to the profile of the membrane of 20–25 Å. In the five-monolayer arrays described here, the individual capacitor distance includes the 57 Å profile of the reaction center [18,19], as well as the spacing of the intervening arachidate monolayer, 26 Å [11], separating each reaction center monolayer, and finally the distance of the four arachidate monolayers separating the monolayer array from each electrode. The charge separation between $(BChl)_2$ and Q_I can then be expected to occur only across about 20% of the individual capacitance, and as a result, the expected current integral for each capacitor will be proportional to this percentage of the number of functioning reaction centers.

From these considerations, the observed $8.62 \cdot 10^{12}$ functioning reaction centers/cm^2 can then be expected to yield a current integral of approx. $5.5 \cdot 10^{-8}$ C/cm^2, if all the reaction centers were aligned and have the electron transfers in the same direction. The largest observed current integral of $6.8 \cdot 10^{-9}$ C/cm^2 is only 12% of this value. This suggests that the reaction centers must be aligned to a variable extent with opposing directions of electron transfer, having at present a maximum of 56% of the reaction centers preferentially oriented with the quinone directed toward the aqueous phase (see Discussion).

Discussion

Previously, approaches for the formation of monolayers of various proteins have been described to permit studies on protein structure through a combination of electron microscopy [13] and infrared spectroscopy by attenuated total reflection [13,14]. In addition, examination of the coupling between protein structure and external electric fields has also been approached by the examination of alterations in infrared spectra of acetylcholinesterase monolayers in the presence of clamped potentials [25].

The work presented here shows that the monolayer technique of Blodgett and Langmuir [10] can be applied to the reaction center protein to construct well oriented reaction center films. However, whether these reaction center films are truly one-molecule-thick monolayers needs to be verified more directly, for example, by visualization with electron microscopy. The density of reaction centers on the air/aqueous interface when compressed to the 30 dyne/cm surface pressure is very close to that predicted for a hexagonal array of 50 Å diameter circles, and suggests that these films may be good approximations to a true reaction center monolayer. This is further supported by at least the variable observation of a transition in the pressure-area curves at this compression. The variability in the pressure-area isotherms indicates that parameters besides the dimensions of the reaction center protein, for example, preparation-dependent variations in the amount of tightly bound phospholipid or detergent to the reaction center, may be critical in determining the packing of the reaction center in the compressed film.

The optical linear dichroism spectra of these films deposited onto slides show little variation in dichroic ratios, demonstrating that the reaction centers consistently tend to align on the air/aqueous interface in the same orientation as they are with respect to the membrane plane in situ. In spite of this, large variations are seen in the extents of the light-induced electrical responses. Since the voltage clamp assays only the net charge separation in a direction perpendicular to the electrode surfaces, the finding that the electrical repsonses are, at best, only 10–12% of that expected for the number of reaction centers observed to be functioning from the flash-induced absorbance changes suggests that reaction centers must be aligned with opposing directions of electron transfer.

This, together with the linear dichroism measurements suggest a structure for the reaction center film in which the reaction centers are aligned on the air/aqueous interface close to the same angular inclination as they are with respect to the membrane plane, but have in the range of 50–60% of the reaction center population oriented in one direction; with the remainder having the same angular inclination, but oriented in the opposite direction. In this series of experiments, the polarity of the light-induced electrical responses demonstrated a tendency for the reaction centers to orient on the air/aqueous interface with the Q_I located near to the aqueous phase, and with the $(BChl)_2$ located nearer to the air phase. The extent of this preferential orientation was very variable, and, in fact, reaction center films having slight enhancement of the opposite functional direction have been seen.

This large variability in whether the Q_I or the $(BChl)_2$ is directed towards the aqueous phase might be expected if the reaction center protein does not have a dominant hydrophobic/hydrophilic polarity. This notion is supported by the observation that the reaction center spans the membrane and can be labeled with antibodies from both sides of the chromatophore membrane [15,26]. The fact that there is a large amount of variability in the extent of the electrical responses suggests that it may be possible to use preparative manipulations to yield films that have a still higher extent of reaction centers functionally pointing in the same direction. For example, one strategy might be to link covalently the reaction center with a highly hydrophilic group, such as cytochrome *c*.

Whether the slight, 'adventitious' asymmetrical arrangement in the direction of electron transfer detected by the voltage clamp results from a true preferential orientation of the reaction center on the air/aqueous interface is difficult to distinguish from the possibility that the asymmetry might be induced by other factors, for example, by the associated lipids or detergents, states of aggregation of the reaction center, or by reorganization while depositing on the tin oxide slide. However, the fact that the direction of electron transfer in the films was dependent upon whether the slide was coated from above or below the interface argues that there is some preferential orientation on the air/aqueous interface. One example of an external factor influencing the functional asymmetry detected by the voltage clamp has been seen when the electrical measurements were made with the mercury or evaporated metal electrodes placed directly on top of the reaction center coating without an arachidate spacer placed in between. In this case, the polarity of the light-induced electrical responses was found to be such that the tin oxide slide became positive irrespective of whether the slide was coated from below or above the air/aqueous interface [22].

The light-induced capacitative charging and discharging currents seen in the reaction center coatings are readily associated with the primary light-induced reaction center chemistry, and are entirely analogous to those generated in the reaction center reconstituted planar bilayers [1–3]. However, the charge density in the single reaction center coatings is a factor of 10–100 greater than that seen in the planar bilayers. The major advantage of the reaction center electrode assemblies lies in the ability to make concomitant electrical and spectrophotometric characterizations on the reaction center films. In spite of the present shortcomings of the reaction center coatings in terms of the incomplete directional alignment and anomalies in partial loss of photochemical activity, these assemblies seem already to present uniquely useful devices for studying the correlations between redox reactions and electrogenic events. For example, these assemblies should provide an opportunity to study electron-transfer rates and electrogenic yield within the reaction center as a function of clamped voltages (imposed electric fields) and temperatures.

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