Architecture and Function of Membrane Proteins in Planar Supported Bilayers: A Study with Photosynthetic Reaction Centers†

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ABSTRACT: We present a simple and convenient method for creating fluid supported bilayers which contain oriented and functional photosynthetic reaction centers (RCs).[1] The supported bilayers are prepared by fusion of proteoliposomes with a glass surface. The proteoliposomes are prepared by spontaneous insertion of RCs into preformed small, unilamellar vesicles. The RCs in these vesicles are shown to be oriented with the cytochrome *c* binding surface on the outside and the H-subunit facing inside. Upon fusion to glass surfaces, the RCs remain functional and highly oriented, with the cytochrome *c* binding surface exposed to the bulk solution. The RCs in the supported bilayers are at a surface density of order 10^{11} RCs/cm^2. The quality of the supported lipid bilayer is characterized by epifluorescence microscopy and the long-range lateral mobility of the lipids by fluorescence recovery after photobleaching. We demonstrate that homogeneous, fluid bilayers can be prepared over large areas (e.g., 1 cm^2) of clean glass surfaces. The lipids in these supported bilayers are laterally mobile, and their diffusion coefficient agrees with values obtained in other fluid bilayer systems. This fluidity is unaffected by the presence of RCs; however, the RCs bearing a site-specific fluorescent label are immobile, despite retaining their charge separation and cytochrome *c* binding properties. We speculate that this results from interactions between the globular domain of the H-subunit and the glass substrate. Because of the unique spectroscopic and functional signatures associated with intact RCs, this system is one of the best characterized examples of a transmembrane protein in a supported bilayer at a nonbiological interface.

Transmembrane proteins in their native state are always uniquely oriented, and their orientation is an essential feature of their function. There are many classes of experiments which require oriented membrane proteins in a simpler lipid bilayer. Although it is often possible to insert membrane proteins into lipid vesicles with a specific orientation, the proteins are not oriented with respect to an external axis because the vesicles are freely diffusing in solution and are spherical. In order to achieve the desired polar or uniaxial orientation, it is necessary to planarize the bilayer and support it in some fashion. Three general approaches have been taken to this problem, and each has been used to study photosynthetic reaction centers (RCs):[1] the Langmuir−Blodgett (LB) technique to prepare monolayers at the air−water interface and build up single or multiple bilayer-type structures (Alegria & Dutton, 1991a,b); the black lipid membrane technique to suspend a single bilayer patch in a small aperture separating two compartments (Gopher et al., 1985); and the "piggyback" approach, in which the membrane protein of interest is bound to a second protein which is itself covalently or electrostatically bound to a surface (Amador et al., 1993). We demonstrate here a fourth technique in which supported bilayers are prepared from proteoliposome vesicles which contain oriented protein. We were motivated in developing this by an interest in preparing uniaxially oriented RCs for nonlinear optical and Stark effect measurements, as a strategy for preorganizing RCs for covalent attachment to glass or electrode surfaces, and as part of a larger program aimed at structurally characterizing and reorganizing biological membranes using applied electric fields (Groves & Boxer, 1995). In the following we describe detailed methods of preparation and characterization of both the lipid and RC protein in supported bilayers which should be applicable to other proteins as well.

The fusion of proteoliposome vesicles with a glass surface to create a planar-supported membrane was first demonstrated by Brian and McConnell (1984). In that work, the H-2K^k protein was reconstituted into egg phosphatidylcholine−cholesterol vesicles by detergent dialysis, and these were used to create a planar membrane on glass. The H-2K^k-containing membrane is useful as a model surface and is capable of eliciting a specific cytotoxic response when brought into contact with a cell. Following that work, Chan et al. (1991) demonstrated that a glycanphosphatidylinositol (GPI) anchored membrane receptor is laterally mobile in planar membranes formed from proteoliposome fusion, and this mobility enhances cell adhesion to the membrane. Other investigators have elaborated on this work by using a combination of vesicle fusion, Langmuir−Blodgett methodology, and derivatized surfaces to prepare supported

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[1] Abbreviations: RC, reaction center; SUV, small unilamellar vesicle; cyt *c*, cytochrome *c*; LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide; LB, Langmuir−Blodgett; DMSO, dimethyl sulfoxide; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; TCEP, tris(carboxyethyl)-phosphine; R-492, rhodamine X iodoacetamide; HABA, 4-hydroxyazobenzene-2′-carboxylic acid; Texas Red DHPE and Texas red, *N*-(Texas Red sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; OD, optical density; bR, bacteriorhodopsin; GPI, glycanphosphatidylinositol.

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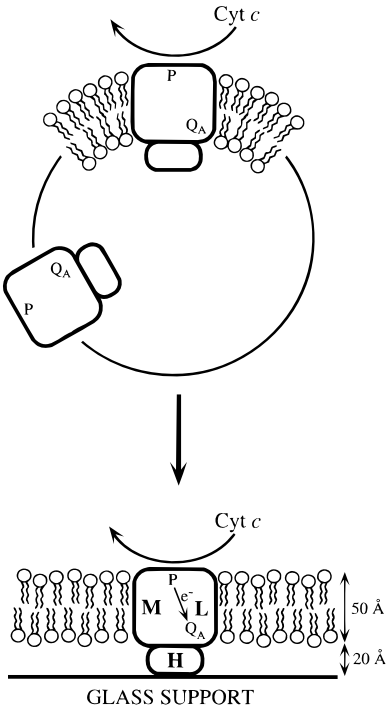


FIGURE 1: Schematic diagram of the bacterial photosynthetic reaction center in a bilayer membrane. The three protein subunits (L, M, and H) and the relevant functional components, the special pair primary electron donor, P, and the primary quinone acceptor, Q_A, are indicated. The RC is depicted in an orientation that is consistent with the results presented and a mechanism in which the vesicles fuse to the glass support by opening out.

membranes (Sui et al., 1988; Plant et al., 1995; Sackmann, 1996), though most of this work does not involve membranes which contain protein.

The RC is the smallest isolatable unit which performs the initial photoinduced charge separation steps in photosynthesis. The three-dimensional structures of RCs from two species of photosynthetic bacteria are known to atomic resolution (Deisenhofer et al., 1995). It is comprised of three subunits, termed L, M, and H, whose total molecular mass is approximately 100 kDa (see Figure 1). Its physiological role is to transduce light energy into a charge separation across the native membrane, which in turn leads to a transmembrane electrochemical potential used by the organism to store energy. The RC accomplishes this charge separation by transferring an electron from the excited state of the special pair primary electron donor, denoted P, to a quinone acceptor, denoted Q_A, to create the internal charge-separated state, $P^+Q_A^-$. The oxidized donor is subsequently reduced by a soluble ferrous cytochrome *c* (cyt *c*) which interacts with the RC at a binding site on the P face of the RC. The sidedness of this cyt *c* reduction step has been widely used to monitor the orientation of RCs in bilayer systems (Pachence et al., 1979; Venturoli et al., 1990). One of the ideal features of the RC is that the kinetics and quantum yield of formation of charge-separated species and the $P^+Q_A^-$ decay kinetics can be sensitively monitored optically, providing a built-in assay for native function. Deletion strains and plasmids for RCs from several species are available (Bylina & Youvan, 1988), so that the RC can be engineered to introduce unique functionality for labeling (Boxer et al., 1992).

EXPERIMENTAL PROCEDURES

Materials. The dye−lipid probes *N*-(Texas Red sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red), and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE), were purchased from Molecular Probes, Inc. (Eugene, OR). The thiol reductant, tris(carboxyethyl)phosphine (TCEP), and the RC conjugate, rhodamine X iodoacetamide (R-492), were also purchased from Molecular Probes. Iodoacetyl-LC−biotin, 4-hydroxyazobenzene-2′-carboxylic acid (HABA), and avidin (Immunopure avidin) were purchased from Pierce Chemical Co. Horse heart cyt *c* was purchased from Sigma Chemical Co.

Reaction Centers. RCs were isolated from *Rhodobacter sphaeroides* wild-type or the R-26 (carotenoidless) strain by methods that are described in detail elsewhere (Debus et al., 1986). The RCs isolated by this procedure generally contain only the primary quinone, Q_A. *Rb. sphaeroides* RCs have five cysteine residues at positions L92, L108, L247, H156, and H234. From an examination of the crystal structure, the cysteine residue at H156 is on the surface of the protein and the other four are buried (i.e., they are more than 10 Å from the surface). *Rhodobacter capsulatus* RCs were isolated as described in Taguchi et al. (1992). These RCs have cysteine residues at positions L92, L98, L108, L246, and L247, all of which are buried assuming the RCs have the same three-dimensional structure as the highly homologous *Rb. sphaeroides* RCs (Komiya et al., 1987). A unique surface cysteine residue was introduced on the P-face of the *Rb. capsulatus* RC by replacing the leucine residue at position M189 to create the strain (M)L189C as described in detail in Boxer et al. (1992).

RC−Biotin and RC−Rhodamine Conjugates of Rb. sphaeroides RCs. The surface of *Rb. sphaeroides* RCs can be modified using reagents which react with free cysteine groups; this was used to create RC−biotin and RC−rhodamine conjugates at the unique surface-accessible cysteine H156 (Debus et al., 1986). *Rb. sphaeroides* R-26 RCs in 10 mM Tris, pH 8.0, and 0.05% LDAO were concentrated to about 75 μM using a Centricon-30 concentrator (Amicon Co.). The RCs were then dialyzed against 10 mM Tris, pH 9.0, and 0.025% LDAO buffer for 1 day, followed by two changes of buffer containing 0.001% LDAO for 1 day each. A 10-fold molar excess of the reductant, TCEP, was added to the RC solution, and the mixture was stirred in a conical vial under nitrogen for 30 min. The conjugate, either iodoacetyl-LC−biotin or the fluorescent dye R-492, dissolved in DMSO, was then added to the mixture through a septum over a 1-min period, to a final label:RC molar ratio of 25 and a final DMSO concentration of less than 10% by volume. The mixture was stirred for 4 h at room temperature in the dark. The excess biotin label was removed by dialyzing each for 1 day against two changes of 10 mM Tris, pH 8.0, buffer containing 0.001% LDAO; the excess dye label was removed by separating it from the RC−dye conjugate using a DEAE ion-exchange column. A control RC sample was treated identically except for the addition of the biotin reagent. The dye:RC molar ratio was determined to be 1:1 from an absorption spectrum of the dye−RC complex, using extinction coefficients of 288 000 M^{-1} cm^{-1} (800 nm) and 82 000 M^{-1} cm^{-1} (590 nm; Molecular Probes catalog) for the RC

and dye, respectively. Absorption from the RC at 590 nm (10% of the 800 nm absorption) was subtracted out for these calculations. A HABA−avidin−dye solution for determining the amount of biotin conjugated to the RCs or present in the proteoliposome samples was made as 0.25 mM HABA and 0.2 mg/mL avidin in 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl (Green, 1970).

Dye Labeling of (M)L189C Rb. capsulatus RCs. Isolated (M)L189C RCs were treated with a 100 molar excess of dithiothreitol at room temperature for 15 min and then concentrated at least twice to deadstop volume in a Centricon to remove the reductant. The protein concentration was adjusted to an OD of 1−2 at 800 nm (3.5−7 μM), and the RCs were incubated for 2 h at room temperature with a 10-fold molar excess of the dye R-492 dissolved in DMSO (the final DMSO concentration was less than 10% by volume). The sample was loaded onto the column and washed with 10 mM Tris, pH 8.0, and 0.1% LDAO to elute the free dye, followed by a salt gradient, with the RC−dye complex eluting at about 300 mM NaCl. This is a higher salt concentration than is required for unmodified RCs, as expected on the basis of the added charge from the attached dye. The dye:RC molar ratio was determined to be 1:1 from the absorption spectrum and extinction coefficients given above.

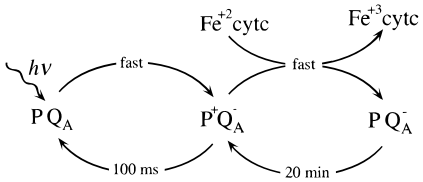
Preparation of Phospholipid Vesicles. Small unilamellar vesicles (SUVs) were prepared by following the protocol outlined in Barenholz et al. (1977) using egg phosphatidylcholine (Sigma). The phosphatidylcholine was mixed with 1 mol % Texas red DHPE in HPLC-grade chloroform (Sigma-Aldrich) and dried in a vacuum desiccator overnight. The dried lipids were resuspended in 10 mM Tris, pH 8.0**,** buffer containing 100 mM NaCl (hereafter referred to as the standard buffer), filtered through Rainin nylon-66 0.45 μm filters using a Sibata filter unit to about 20 mg/mL, and sonicated to clarity with a Branson ultrasonicator under flowing Ar on ice for 3 min periods separated by 1 min cooling periods. The sample was then spun for 30 min at 100000*g* to remove Ti particles from the sonicator tip, and the supernatant was spun for 4 h at 166000*g* to obtain the SUVs. The SUVs were stored at 4 °C under N_2 or Ar in the dark and were used within 3 weeks. The lipid concentration in these samples was determined from the Texas Red probe absorption at 590 nm (ϵ = 100 000 M^{-1} cm^{-1}; Molecular Probes catalog, 1994), assuming that the probe concentration in the vesicles is 1 mol % as prepared. Yields (milligrams of SUV lipid per milligram of initial lipid) were calculated from this concentration and are equal to those reported by Barenholz et al. (1977).

Preparation of Proteoliposomes. Proteoliposomes were prepared according to the protocol developed by Sadler et al. (1984). RCs were concentrated to 6−11 OD (800 nm) in 10 mM Tris, pH 8.0, and 0.1% LDAO buffer using a DEAE ion-exchange column or Centricon concentrator, and the salt concentration was adjusted to 100 mM NaCl by dialysis. The RCs were added to the SUVs in a small conical-bottom vial with stirring, typically to a final RC:lipid mole ratio of 1:350. Samples were then run down a Sepharose CL-4B (Sigma Co.) column, previously equilibrated with SUVs to minimize lipid adsorption, and fractions were collected. The absorption spectra of the proteoliposome fractions were measured, and the RC:lipid mole ratio was calculated using the absorption peak of the Texas red lipid label. Typically, the mole ratio of RC:lipid in the fractions follows a monotonic decrease, beginning at about 1:300 and ending with 1:1000−1200. Only the fractions with a mole ratio of about 1:500 or lower are used to make planar-supported bilayers; the fractions with higher mole ratios do not always form uniform planar bilayers.

Cytochrome c Trapping in SUVs and Proteoliposomes. Horse heart cytochrome *c* was trapped inside SUVs by sonicating the lipids in the presence of 8 mM cyt *c*. The cyt *c* was prereduced with sodium dithionite, and the reductant was removed with a Sephadex G-50 (Sigma) filtration column. Cyt *c* external to the SUVs was separated from the SUVs with trapped cyt *c* on a Sepharose CL-4B column previously equilibrated with SUVs to minimize lipid adsorption. No further change in the cyt *c* content was observed upon running these SUVs down a second CL-4B column, demonstrating that the cyt *c* is stably trapped inside. Proteoliposomes with trapped cyt *c* were prepared by using these SUVs with trapped cyt *c*. A control was performed to demonstrate that added cyt *c* could be separated from proteoliposomes on a CL-4B column.

Function and Orientation of RCs in Proteoliposomes: P^+Q^- Recombination Kinetics. The $P^+Q_A^-$ recombination kinetics in RCs were measured at room temperature by exciting the samples with a saturating 10 ns 532 nm YAG laser pulse gated at 2 Hz. The change in absorption was probed at 865 nm using light from a 100 W tungsten bulb, powered by a 12 V regulated supply (Model PAL16−20, Kikusui Co., Yokohama, Japan), passed through a single monochromator with a resultant bandwidth of about 1 nm. The probe light intensity at the sample was 10 $\mu W/cm^2$. The change in absorption was detected with a home-built detector using an HUV-1100BG photodiode (EG&G Co.) with a measured response time of 1 ms (adequate for the 100 ms $P^+Q_A^-$ decay while minimizing higher frequency noise).

The orientation of the RCs was measured by observing whether ferrous cyt *c* reduces P^+ as outlined in Pachence et al. (1977). The relevant reaction scheme and kinetics are



Under the conditions of the experiments, the bimolecular rate constant for ferrous cyt *c* reduction of P^+ is roughly 1 × 10^7 M^{-1} s^{-1} (Overfield & Wraight, 1980). Given this rate constant and the concentrations of RCs and cyt *c* used in our kinetics experiments (35 nM RCs; 2−3 μM ferrous cyt *c*), virtually all of the RCs with accessible P-faces in the proteoliposomes will be reduced by the cyt *c* within 15 ms. By probing the amplitude of the P^+ decay with and without cyt *c*, the accessibility of the P-face can be determined.

Preparation and Characterization of Planar Supported Bilayers Containing RCs. The planar bilayers were formed by exposing a clean glass coverslip (Corning cover glass, 18 mm square; cleaned by rinsing with distilled water and then baking at 450 °C for 5−8 h) to a sample of the proteoliposomes (lipid concentration 0.1 mg/mL or higher) for 5−10 min. The resulting supported bilayer must be kept hydrated at all times and was destroyed if exposed to air.

Three methods were used for making the supported bilayers: (i) For fluorescence microscopy studies, the supported bilayer was made on one side only by dropping the coverslip onto a 100 μL drop of proteoliposome solution in a clean petri dish. After incubating, the excess proteoliposome solution was washed away with the standard buffer, and the coverslip was transferred under the same buffer to a microscope slide with the supported bilayer side facing into the well to keep the bilayer hydrated. (ii) For absorption spectroscopy, both sides of the square coverslip were coated with supported bilayer by immersion in a Teflon trough with 1 mm wide slits cut to a depth of half the height of the coverslip. This produces supported bilayer on both sides of a coverslip, but only half-coated on each side. Eleven glass coverslips were placed into a slotted Teflon holder with 1 mm between each surface and immersed in the standard buffer in a 30 mm square cuvette (Wilmad Glass Co.). An absorption baseline was obtained by scanning the uncoated portion against air with a spectral slit width of 2 nm, at a scan rate of 960 nm/min. The cuvette was then carefully translated in the spectrometer compartment to the supported bilayer side of the coverslips; this side was scanned as the sample. Averages were made by averaging separately made scans. (iii) For $P^+Q_A^-$ recombination kinetics measurements, the coverslips were fully coated with supported bilayer by complete immersion in a Teflon trough.

The $P^+Q_A^-$ recombination kinetics of RCs in supported bilayers were measured using the same setup described above for the proteoliposomes, except the probe light was detected with a home-built two-stage capacitively coupled detector (Joe Rolfe, Stanford University electronics shop). Eleven glass coverslips, fully coated on both sides with bilayer, were placed into the Teflon holder in the 30 mm^2 cuvette and were excited at an angle of about 20° to the probe beam. Prereduced cyt *c* was added by immersing the square cuvette in a bath containing a solution of this protein.

The uniformity and fluidity of the supported bilayers were monitored by epifluorescence microscopy (Nikon Labophot microscope with rhodamine and fluorescein filters) using either the fluorescent dye−lipid probe or fluorescently labeled RCs. Observations of the bilayer were made with the 10× or 40× objectives giving essentially diffraction-limited resolution of the membrane. Photographs of bilayer fluorescence were taken with a Nikon F3 camera mounted on the microscope. Photobleaching of the bilayer was accomplished by illuminating a portion of the membrane continuously with the excitation light; a circular region was bleached by shadowing with the microscope aperture.

Moles of Lipid in Supported Bilayers Containing RCs. The lipid content of the supported membranes was determined by quantitative fluorescence measurements of the resolubilized films. Membranes were dissolved off the surface into a known volume of the standard buffer with 0.1% LDAO for fluorescence measurements. Standard solutions were made by dissolving known amounts of the same lipid mixture used to make SUVs into the 0.1% LDAO buffer. The fluorescence intensity at 610 nm of the dye−lipid probe in this solution was measured with a fluorometer and calibrated against that of a standard using a square 1 cm path-length cuvette in front-face collection mode. These measurements confirm that vesicle fusion consistently produces single supported bilayers.

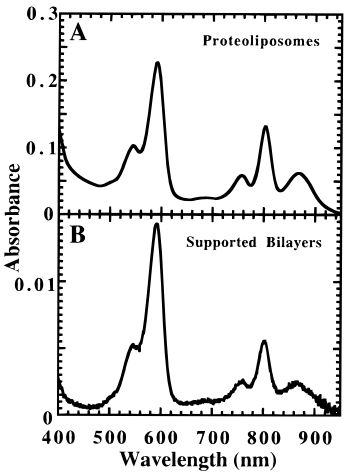


FIGURE 2: (A) Absorption spectrum of RC-containing proteoliposomes in the standard buffer at room temperature. The lipid contains 1 mol % Texas Red DHPE whose absorption maximum occurs at 590 nm and from which the RC:lipid ratio can be obtained (1:500 for the case shown). Comparing the magnitude of this peak with the 800 nm peak of the RC allows calculation of the RC:lipid ratio. The distinctive set of three absorption peaks from 700 to 900 nm is due to bacteriochlorophyll and bacteriopheophytin cofactors in the RC and is sensitive to the structural integrity of the RC complex. (B) Absorption spectrum of 11 glass coverslips, each coated on both sides with supported lipid bilayers containing RCs, measured at room temperature in the standard buffer (average of two scans). The magnitude of the RC absorption indicates an RC surface density on the order of 10^{11} RCs/cm^2 and a surface coverage of 10%; the magnitude of the absorption peak at 590 nm due to the Texas Red DHPE dye−lipid probe is consistent with a single bilayer architecture.

RESULTS

Absorption Spectrum of Proteoliposomes. A typical absorption spectrum of the proteoliposomes is shown in Figure 2A. The ratio of the absorption of the dye-labeled lipid to the RCs is used to calculate the molar ratio between the lipids and the RC. Ratios vary with proteoliposome fraction from 1:350 to 1:1200 (the sample shown is 1:500). At this mole ratio, the surface area coverage of the RCs in the vesicle bilayer is about 10%, assuming an area of 70 Å^2 occupied by a lipid molecule (Gennis, 1989) and 2500 Å^2 (Yeates et al., 1987) occupied by the RC.

Absorption Spectrum of RCs in Supported RC Bilayers. The absorption spectrum of RCs in supported lipid bilayers on glass was measured to provide four important pieces of information. First, the line shape demonstrates that the RCs are structurally intact in the supported bilayers as shown in Figure 2B. Second, the magnitude of the RC absorption indicates the RC coverage is of the order of 10^{11} RCs/cm^2. For most samples, the RC:lipid mole ratio is 1:550, which corresponds to an RC surface coverage of 10%. Third, the magnitude of the dye−lipid absorption indicates a single lipid bilayer. The single bilayer nature of the supported membranes is further confirmed by quantitative fluorescence of the resolubilized membranes. Fourth, the mole ratio of RCs to lipid in the supported bilayers is approximately equal to that of the proteoliposomes used to make them; the agreement is more exact when more homogeneous proteoliposome fractions are deposited. This indicates that the glass-supported structure is a single bilayer formed from the vesicle bilayer and that the RCs remain associated with the bilayer during the fusion process.

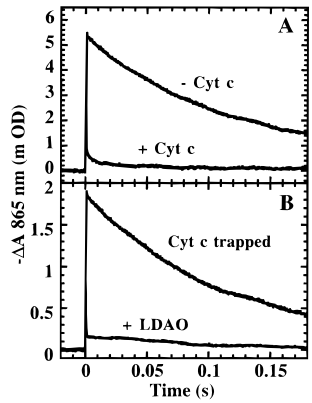


FIGURE 3: (A) $P^+Q_A^-$ recombination kinetics of RCs in proteoliposomes without and with addition of ferrous cytochrome *c* in the standard buffer measured at room temperature. The concentrations of RCs and cyt *c* are 35 nM and 2 μM, respectively. The traces are an average of 700 shots. The data demonstrate that the protein is functional and oriented in proteoliposomes with the P-side facing outward. (B) $P^+Q_A^-$ recombination kinetics of RCs in proteoliposomes with ferrous cytochrome *c* trapped inside and upon disruption of the proteoliposomes by addition of 0.01% LDAO in the standard buffer at room temperature. The concentrations of RCs and cyt *c* are 27 and 200 nM, respectively. The traces are an average of 700 shots. The data demonstrate that the lipid structures are vesicular and that trapped cyt *c* cannot access the P-face (cyt *c* binding site) of the RCs in proteoliposomes.

Function and Orientation of RCs in Proteoliposomes. Three experiments are presented to demonstrate that the RCs are embedded in the vesicle lipid bilayer and are oriented with the P-face out and the Q-face (H-subunit) facing inward as illustrated in Figure 1. One experiment demonstrates that the P-face of the RCs in proteoliposomes is accessible to the external solution; a second experiment demonstrates that the vesicles are capable of trapping cyt *c* and that the RC P-face is not accessible to the trapped cyt *c*; a third experiment shows that the H-subunit is not accessible to the external solution using a specific and unique biotin label.

First, the percentage of RCs with accessible cyt *c* binding sites was determined using the bleach amplitudes of $P^+Q_A^-$ recombination kinetics with and without added cyt *c*, as described by Pachence et al. (1979). Briefly, this is determined from the ratio of the bleach amplitude with added cyt *c* to the bleach amplitude without cyt *c* following excitation with a saturating flash. A single flash was used to measure the rate of reduction of the bleach amplitude of P^+ by cyt *c* to determine that all of the bleach amplitude is eliminated by cyt *c* within 20 ms, as expected given the known rate constant and reactant concentrations (Overfield & Wraight, 1980). Multiple flash data were then taken and the bleach amplitudes were used to determine the accessibility of the P-face of the RCs in the proteoliposomes. The results of these experiments are shown in Figure 3A. The data were fit to a single exponential to determine a rate constant of 7.2 s^{-1} for $P^+Q_A^-$ recombination, which demonstrates that the RCs are functional in proteoliposomes (Pachence et al., 1979). From the ratio of the bleach amplitudes at 20 ms for the traces without and with added cyt *c*, the percentage of RCs in proteoliposomes with the cyt *c* binding site accessible is 95%.

Second, we demonstrate that the lipid structures are vesicular and assay for the percentage of RCs with cyt *c* binding sites exposed to the interior of the vesicles by trapping cyt *c* within SUVs and proteoliposomes. The cyt *c* is first trapped within SUVs, and these are used to make proteoliposomes. An absorption spectrum of proteoliposomes with trapped cyt *c* was measured to determine the cyt *c*:RC mole ratio, which was found to be about 6:1 using the known extinction coefficients of each (data not shown). This and the observation that vesicles could be passed through a sizing column without loss of trapped cyt *c* demonstrate that stable vesicular structures are present. The $P^+Q_A^-$ recombination kinetics of RCs in proteoliposomes with trapped cyt *c* is shown in Figure 3B. The bleach amplitude is within 5% of that measured on an OD-matched sample of detergent-solubilized RCs in the absence of cyt *c*. This demonstrates that 95% of the cyt *c* binding face is oriented outward (binding of trapped cyt *c* to lipids could interfere with binding to RCs, but the cyt *c* exhibits negligible binding to egg phosphatidylcholine vesicles (Rytomaa et al., 1992; Gennis, 1989). Addition of LDAO (0.01%) disrupts the vesicles releasing the cyt *c* which can then bind to RCs. As shown in Figure 3B, this reduces the bleach amplitude of the recombination kinetics. The extent of the bleach amplitude reduction is determined by the number of cyt *c* molecules in the reduced state per RC.

From the results of the first two orientation experiments, the RCs are either embedded in the vesicle bilayer with the H-subunit facing inward as illustrated in Figure 1 or they are associated with the vesicles in some nonspecific way outside of them. The third experiment was designed to resolve these two alternatives as follows. A molecule of biotin was bound covalently to the unique cysteine residue at position (H)156 in *Rb. sphaeroides* RCs (Debus et al., 1986), creating a label for probing the H-subunit accessibility in proteoliposomes. The biotin is colorimetrically detected using a HABA−avidin solution as described by Pierce (Pierce Chemical Co. catalog). Briefly, the HABA dye binds to the biotin-binding sites in avidin; it has a large absorption at 500 nm in this state and a very small absorption in the free state (difference in extinction coefficient at 500 nm between the two states is 34 000 M^{-1} cm^{-1}). Added biotin (free or covalently bound to protein) will quantitatively displace the bound HABA, and the resultant decrease in absorption at 500 nm can be used to determine the amount of added biotin.

The HABA−avidin solution was placed into cuvettes in both the sample and reference paths of the spectrometer, and a baseline was measured. The biotin-labeled RCs or unlabeled control RCs were then added to the sample cuvette, and the absorption spectrum was measured as shown in Figure 4A. From the difference in absorption at 500 nm of the biotin-labeled RCs and control RCs, the biotin:RC molar ratio can be determined. The molar ratio is determined using the absorption at 800 nm (extinction coefficient of 288 000 M^{-1} cm^{-1} for the RCs) and the difference in absorption between the biotin-labeled and control RCs of the HABA dye at 500 nm (extinction coefficient difference of 34 000 M^{-1} cm^{-1}). From this analysis, the molar ratio of biotin:RC is 1:1, which is consistent with the label bound to the single, free cysteine at H-156.

Proteoliposomes were then prepared using the biotin-labeled RCs, and these were added to the HABA−avidin solution to test for the presence of biotin as shown in Figure 4B. There is no change in the absorption at 500 nm, which indicates that there is no exposed biotin in the proteoliposomes with biotin-labeled RCs, consistent with the RCs being

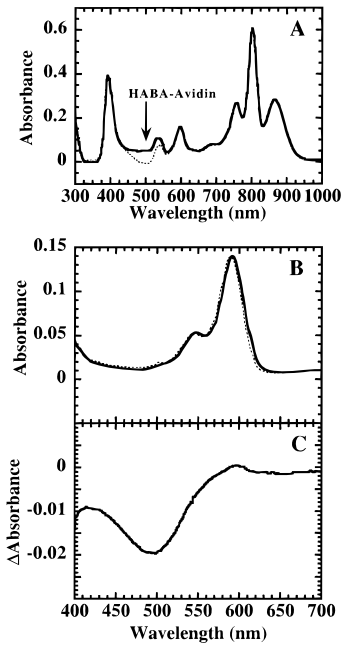


FIGURE 4: (A) Absorption spectra of biotin-labeled RCs (labeled on the H-subunit) (---) and control (unlabeled) RCs (—) added to the HABA−avidin solution. A baseline was measured with HABA−avidin solution alone. (B) Absorption spectrum of RC−biotin proteoliposomes (—) and RC proteoliposomes (---) added to the HABA−avidin solution. The spectra were normalized at 590 nm. (C) Difference absorption spectrum of biotin−RC proteoliposomes against RC proteoliposomes with trace LDAO (0.001%) added to each in HABA−avidin solution. The baseline is the absorption due to the biotin−RC and RC proteoliposomes in the HABA−avidin solution.

embedded in the vesicles with the H-subunit facing inward. A control was performed to demonstrate that RC (without biotin) proteoliposomes do not produce a signal for biotin when added to the HABA−avidin solution; this is shown in Figure 4B. Finally, the difference absorption spectrum of the biotin-labeled RCs in proteoliposomes and unlabeled RCs in proteoliposomes with trace addition of LDAO (0.001%) to disrupt the vesicles is shown in Figure 4C. There is no further change in the absorption upon addition of a second aliquot of LDAO. The signal is of the expected magnitude (using the measured concentration of RCs in these samples, a molar biotin:RC ratio of 1:1, and a difference extinction coefficient of 34 000 M^{-1} cm^{-1} for the HABA dye) and confirms that the RCs are embedded in the vesicle lipid bilayer and are oriented with the H-subunit facing the interior.

Function and Orientation of RCs in Supported RC Bilayers: P^+Q^- Recombination Kinetics. The $P^+Q_A^-$ recombination kinetics of the RCs in supported bilayers were measured to demonstrate the functionality of the RCs as shown in Figure 5.

The decay kinetics were fit to a single exponential with a rate constant of 7.9 s^{-1}, which demonstrates that the RCs are functional (Pachence et al., 1979). Addition of cytochrome *c* results in a disappearance of the bleach amplitude of the millisecond recombination kinetics, demonstrating that more than 90% of the RCs are oriented in the supported bilayer with the P-face accessible to the bulk solution. The cyt *c* was then removed by successive buffer dilution, and the bleach amplitude of the RCs was found to return to its original amplitude within 2 h. This is consistent with the time reported elsewhere (Gopher et al., 1985) for recovery

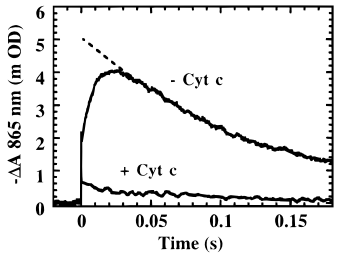


FIGURE 5: $P^+Q_A^-$ recombination kinetics (solid line) and single exponential fits to the data after 50 ms (dashed line, see text) of 11 glass cover slips with supported lipid bilayers containing RCs in the absence and upon addition of 10 μM ferrous cyt *c*. The recombination time of the kinetics in the absence of cyt *c* demonstrates that the RCs are functional in supported bilayers. Nearly complete loss of the bleach amplitude upon addition of cyt *c* indicates the RCs are oriented with the P-side (cyt *c* binding site) facing bulk solution.

of the PQ_A^- state to the PQ_A state, where reversible charge separation to produce $P^+Q_A^-$ can then occur again in the absence of cyt *c*. The supported bilayers were uniform and unchanged upon addition of the cyt *c*, as observed by fluorescence microscopy of the fluorescent dye−lipid probe. The initial sharp rise in the bleach amplitude is due to scattered excitation light into the detector, and the initial round off in the decay is due to the instrument response of the detector which truncates the initial bleach amplitude by 20%. The detector response due to the scattered excitation light decays to the preflash baseline with 50 ms; the kinetic traces were fit using data after this initial 50 ms artifact, and these fits were used to obtain the initial bleach amplitude.

Fluorescence Imaging of Lipids in Supported Bilayers Containing RCs. Visualization of supported membranes using epifluorescence microscopy was also an invaluable test of membrane quality and was used as a check for bilayer formation after each deposition. The bilayer was scratched with the tip of a pair of tweezers, which removes bilayer creating boundaries to lateral diffusion. A bilayer of high integrity appears as a fairly uniform field of fluorescence; a typical example is shown in Figure 6A. A nonuniform bilayer appears as a field of fluorescence with dark spots or patches distributed throughout the field, while failure to form bilayers appears as a field of bits of fluorescence separated by dark space between unfused vesicles, visible as brighter dots of fluorescence. Vesicles are not adsorbed to the planar membranes. Generally, the proteoliposome fractions with lower RC:lipid mole ratios (<1:700) give the most uniform planar bilayers.

The bilayer lipids are laterally mobile, as demonstrated by photobleaching a small spot of the dye−lipid using the microscope aperture and watching the fluorescence within this spot recover with time. We assume that the mobility of the dye−lipid indicates general membrane mobility. Figure 6A depicts a photobleached spot shortly after bleaching; the same region is shown after 20 min in Figure 6B. Complete fluorescence recovery after photobleaching of regions outside the enclosed triangle indicates all lipids are mobile over long distances; no immobile fraction is observed. Trapping of bleached dye−lipid within the triangle demonstrates the lateral nature of the lipid mobility; the dye−lipid is not partitioning into the bulk solution, which would result in some recovery in this region.

Crude estimates of the diffusion coefficient were obtained by observing the time scale of the recovery of a spot

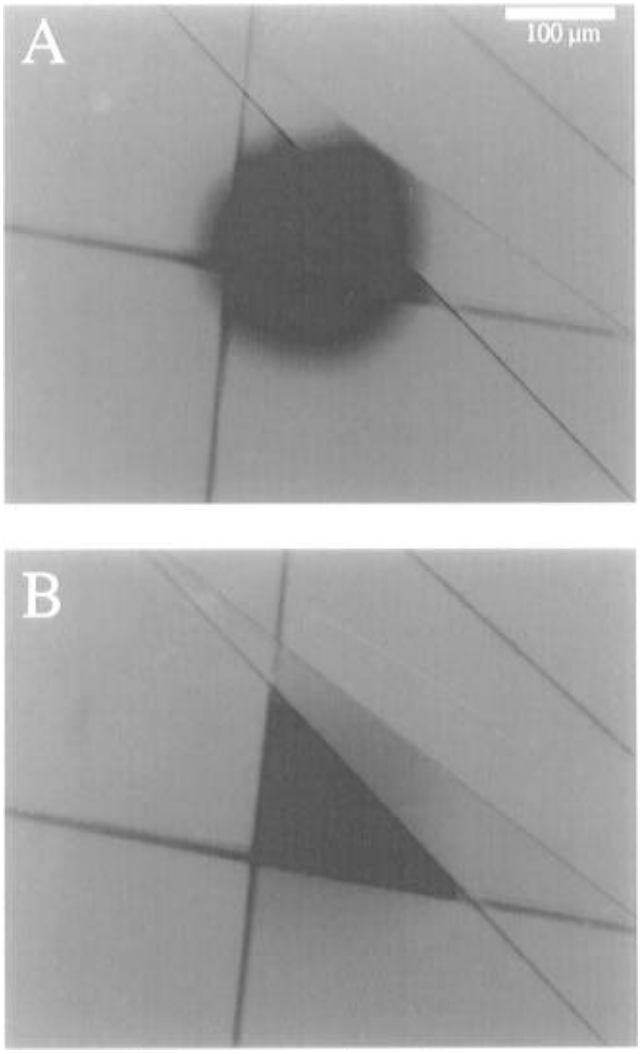


FIGURE 6: (A) Photograph of fluorescence from supported bilayers containing RCs on glass. The mole ratio of RC:lipid in the sample shown is about 1:700. One mole percent of Texas Red DHPE was used as the fluorescent lipid. The sharp dark lines are scratches in the lipid bilayer surface that create physical boundaries to lateral diffusion of the lipids. Prior to being scratched, the surface appeared homogeneous. A circular region of the lipid was shadowed out by the microscope aperture and photobleached with the excitation light. (B) The same field of view 20 min after photobleaching. The photobleached area now fills the central bounded triangular region; other unbounded regions outside the triangle have recovered a substantial amount of fluorescence, as long-range lateral diffusion of fluorescent and photobleached lipid leads to mixing. These results demonstrate that the lipid component is freely diffusing in two dimensions. The fact that bounded regions remain bleached indicates that dye−lipid partitioning into the aqueous phase and spontaneous fluorescence recovery are negligible.

unhindered by scratch boundaries. The diffusion coefficient, D, is related to the half-life of this recovery: $D = r^2/4\tau$ (Sackmann, 1980), where r is the radius of the spot and τ is the time it takes for half of the integrated fluorescence intensity to return. A spot 200 μm in diameter shows significant recovery in 5−10 min, indicating that τ is within this range giving diffusion coefficients of 4−8 $\mu m^2/s$, which is in agreement with the values found by others in more quantitative experiments [4.4 ± 0.5 $\mu m^2/s$; see Stelzle et al. (1992)].

Fluorescence Imaging of Labeled RCs in Supported Bilayers Containing RCs. *Rb. sphaeroides* RCs covalently labeled at cysteine (H)156 (the H-subunit side) with the cysteine-reactive fluorescent rhodamine dye R-492 were used for imaging the protein component in the supported bilayers. These supported bilayers were doped with 1 mol % of NBD-PE instead of the Texas red lipid label, due to overlap of the fluorescence spectrum of the Texas red label with that of R-492. The fluorescence intensity of the labeled RCs in supported bilayers is uniform across the surface of the glass and exactly matches the scratch patterns of the fluorescent dye−lipid probe for a given sample. Observations of the fluorescence intensity were made over a 30 min period following bleaching of a roughly 100 μm diameter spot. Unlike the lipids described above, the RCs exhibit no lateral diffusion on this time scale. Diffusion coefficients of fluid protein components in a natural membrane have values between 1 and 5 $\mu m^2/s$ (Gennis, 1989), so the labeled RCs in the supported bilayers are essentially immobile.

An identical experiment was performed with the *Rb. capsulatus* RC mutant (M)189LC, labeled with the fluorescent dye R-492. This experiment was performed to investigate whether the lack of mobility of the H-subunit labeled *Rb. sphaeroides* RCs might be due to interactions between the fluorescent label and the glass substrate (cf. Figure 1). The engineered cysteine is expected to be the only free, surface cysteine (H-156 cysteine is absent in the *Rb. capsulatus* RC) from a consideration of the crystal structure of the *Rb. sphaeroides* RC and its high sequence homology with the *Rb. capsulatus* RCs (Komiya et al., 1987); this is verified experimentally, as the resultant dye−RC conjugate has a molar ratio of dye to RC of 1:1. The position of the engineered cysteine is situated in the binding site of the cyt *c*, on the opposite side of the RC from the H-subunit. No mobility of these RCs was observed in supported bilayers.

DISCUSSION

Our goal in this work was to create an architecture with oriented RCs on a solid support. The results demonstrate that RC-containing proteoliposomes can be created from preformed SUVs in a highly asymmetric way with the cytochrome *c* binding site facing outward. This is consistent with the result reported by Sadler et al. (1984) but is further substantiated by the biotin labeling experiments which, along with the cyt *c* binding experiments, demonstrate that the RC is genuinely oriented in a trans-bilayer configuration.

The RCs remain oriented in supported bilayers with the cytochrome *c* binding site facing the bulk solution, as illustrated schematically in Figure 1. This indicates that vesicle fusion occurs in a way which opens the side of the vesicles near the glass surface, followed by a flattening of the vesicles from a spherical to a planar shape. Other evidence for this fusion mechanism can be found in the work of Contino et al. (1994). It is conceivable that one could direct the orientation of a protein in proteoliposomes and, consequently, in supported bilayers by binding charged molecules to the protein's surface or modifying it in some other way.

There is evidence that the distribution of charges on the surface of a protein determines its orientation in proteoliposomes. For example, Steverding et al. (1990) have shown that the net orientation of bovine heart cytochrome *c* oxidase can be changed from one direction to the other by chemically modifying the lysine amino groups to make them negatively charged; however, in neither case is the net

orientation greater than 40%. The issue of orientation in reconstitution has also been studied using bacteriorhodopsin (bR) as a model protein and by varying detergent type, concentration, and removal method (Rigaud et al., 1987). An important conclusion is that when bR was incorporated into preformed vesicles, it orients unidirectionally.

The observation that RCs in the supported bilayers are immobile, while the lipids are fully fluid, suggests that the protein interacts with the glass surface. In the supported membranes described here, the RCs are oriented with the H-subunit facing toward the glass surface. Packing models of a lipid bilayer around the RCs indicate that the H-subunit domain projects roughly 20 Å outside of the bilayer (Deisenhofer et al., 1984; Yeates et al., 1987), which is the approximate distance found between the membrane surface and the support for pure supported bilayers on glass (Bayerl & Bloom, 1990) and quartz (Johnson et al., 1991). The transmembrane protein H-2K^k (Brian et al., 1984) and the transmembrane isoform of LFA-3 (lymphocyte function-associated antigen 3) were also found to be immobile in supported bilayers (Chan et al., 1991). In contrast, highly mobile proteins associated with supported lipid bilayers can be made if they are tethered to the membrane by a GPI linker (Chan et al., 1991; Groves et al., 1996).

Preliminary studies using the transparent semiconductor indium−tin oxide (ITO) indicate that vesicle fusion can be used to form an organized interface between RCs and this material. Proteoliposomes spontaneously fuse with the naturally hydrophilic ITO surface to form a uniform layer as observed by epifluorescence microscopy. Quantitative fluorescence of the resolubilized film suggests that the lipid content is consistent with single bilayer coverage as for glass supports. In contrast to glass-supported bilayers, both the lipid and RC protein were immobile on ITO as seen by the failure of photobleached spots to recover fluorescence. Absorption spectra indicate that the RCs are structurally intact. Measurements of the $P^+Q_A^-$ charge recombination kinetics were compromised by absorption and scatter from the ITO film itself. Rough data confirm that the RCs are functional and at least 80% oriented with the same orientation as on glass.

The supported bilayer system made by fusion of proteoliposomes is an attractive alternative to other membrane systems. Langmuir−Blodgett (LB) techniques are highly sensitive to experimental conditions and labor intensive. Black lipid membranes (BLMs) closely resemble lipid bilayers and are well suited to electrical measurements under near physiological conditions, but they are not so easily manipulated or mechanically robust as supported bilayers. It is also difficult to control or measure the amount of protein incorporated into BLMs. Furthermore, supported bilayers can be prepared over large areas (>cm^2) unlike the BLMs (typical area of 0.01 cm^2). The RCs are also oriented unidirectionally by the SUV−proteoliposome method rather than bidirectionally with the BLM (Gopher et al., 1985). The use of self-assembled monolayers to bind detergent-solubilized proteins directly has not been reported and is likely quite sensitive to detergent−surface interactions. Derivatized surfaces which bind monolayers of cytochrome *c* to which RCs have been bound have been reported (Amador et al., 1993); however, in this case both the cytochrome *c* and RCs are adsorbed to the surface at densities significantly higher than close packed (twice and three to four times, respectively), which suggests that the protein−surface architecture is not clearly defined.

In conclusion, we have demonstrated the use of a vesicle fusion method to create planar-supported bilayers which contain oriented and fully functional RCs. The RCs were first reconstituted into proteoliposomes with unidirectional orientation by insertion into preformed SUVs. The structural integrity, functionality, and orientation of the RCs are preserved when the proteoliposomes are deposited as supported bilayers.

This work provides a uniquely well-defined picture of the supported protein−lipid bilayer architecture. The RC-containing supported membranes are stable for at least weeks, a testament to the native-like environment provided by the architecture. The results presented here, along with the convenience and generality of the vesicle fusion method, suggest that supported bilayers may be useful in a much wider range of applications.

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