Biochemistry

 Volume 19, Number 8 April 15, 1980

Reaction Center–Phospholipid Complex in Organic Solvents: Formation and Properties†

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ABSTRACT: A complex between soybean phospholipids and purified reaction centers (RCs) from *Rhodopseudomonas sphaeroides* R-26 was formed in organic solvents. The complex is the starting material for the incorporation of RCs into model membranes. The preparative procedure includes four basic steps: (a) dilution of the detergent used to solubilize the RCs in water; (b) association of RCs with phospholipids by sonication; (c) neutralization of excess negative charges by magnesium ions; (d) extraction of the complex into hexane. Magnesium could be replaced by calcium, and hexane could be replaced by other alkanes but not by more polar organic solvents. The optical absorbance spectrum of the hexane extract, in the visible and infrared regions, was similar to that of purified RCs in aqueous detergent solution. The extract was stable for several weeks when stored at 4 °C in darkness. The molar ratios phospholipid/RC and detergent/RC in the extract were about 6000 and 400, respectively. The size of the RC–lipid complex was determined by Nuclepore filtration to be between 0.015 and 0.08 μm. The complex sedimented by centrifugation at 110000*g* for 1 h. Repeated washings of the sedimented complex with hexane reduced the lipid and detergent content by up to 2 orders of magnitude. The hexane extract, when supplemented with an excess of ubiquinone 10, exhibited light-induced optical and EPR absorbance changes which are typical of RCs in aqueous detergent solution. The low-temperature kinetics of the back reaction in the dark were found to be similar to those determined for RCs in the chromatophore membrane or when purified. The RC–lipid complex, dispersed in water after evaporation of the hexane, showed full photoactivity with no need for quinone addition. Diaminodurene reduced photooxidized RCs in hexane and also (in its oxidized state) replaced the extracted quinone as an electron acceptor. Several other lipid-soluble redox compounds mediated electron transfer reactions with RCs in the organic solvent. Thus, RCs retain their characteristic photochemical activity in the organic solvent and, therefore, can be used for functional reconstitution studies in planar bilayers and other model membranes.

The progress achieved during the last years in the isolation and characterization of the photosynthetic reaction center protein (RC)[1] from bacteria brought with it a better understanding of the primary event in photosynthesis [for a review, see Clayton & Sistrom (1978)]. In the primary event, light energy is used to transfer an electron, thereby creating a charge separation against a redox potential difference. According to the chemiosmotic theory (Mitchell, 1968), this charge separation occurs across the membrane and produces an electric field that plays a crucial role in ATP synthesis. There is evidence that the RC spans the plasma membrane (Prince et al., 1975; Feher & Okamura, 1977; Valkirs, personal communication). Thus, the RC, when incorporated and properly oriented in a lipid bilayer, may constitute the minimum photosynthetic entity that can generate a light-induced electric field. The use of *planar* bilayers affords one the possibility of studying the photochemical reactions by direct electrical measurements.

A current method to incorporate proteins into planar lipid bilayers requires as an intermediate step the formation of a protein–lipid complex in an organic solvent (Montal, 1974, 1976). In this work, we describe a procedure developed to produce such a complex. It is largely based on methods previously developed for other membrane proteins (Das & Crane, 1964; Gitler & Montal, 1972; Montal & Korenbrot, 1973; Montal, 1974; Darszon et al., 1978). The preparative procedure was optimized with respect to the extraction yield of the RC–lipid complex in hexane and to its spectral integrity. The properties of the RC–lipid complex, i.e., its composition, size, and stability, were studied. The photoactivity of the complex was determined by optical and EPR spectroscopy. A preliminary account of this research has appeared (Schönfeld et al., 1979a). While this work was in progress,

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[1] Abbreviations used: RC(s), reaction center(s); Q-10, ubiquinone 10; DAD, diaminodurene; LDAO, lauryldimethylamine oxide; EDTA, ethylenediaminetetraacetic acid; TL buffer, 10 mM Tris, pH 8, and 0.025% LDAO; D, primary donor; A, primary acceptor.

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we were informed of similar research being performed in another laboratory (Kendall-Tobias & Crofts, 1979; Kendall-Tobias, 1979).

Materials and Methods

The following materials were obtained from the indicated sources: diaminodurene, Aldrich Chemical Co., Inc., Milwaukee, WI; α-L-lecithin from soybeans and ubiquinone 10, Sigma Chemicals, St. Louis, MO; LDAO, Onyx Chemical Co., Jersey City, NJ; spectroquality *N*-alkanes, Mallinckrodt, Inc., St. Louis, MO; [^{3}H]H_2O (5 mCi/mL), Amersham, Arlington Heights, IL; [^{14}C]LDAO (3.4 mCi/mmol), ICN Corp., Irvine, CA; dioleylphosphatidylcholine, Applied Science, State College, PA; Bio-Beads SM-2, Bio-Rad Laboratories, Richmond, CA; Nuclepore filters, Nuclepore Corp., Pleasanton, CA.

Reaction Centers. RCs were isolated and purified from *Rhodopseudomonas sphaeroides* R-26 as previously described (Feher & Okamura, 1978). Their concentration was determined by their absorbance at 802 nm (ϵ_{802} = 288 mM^{-1} cm^{-1}; Straley et al., 1973). One-milliliter aliquots of RCs ($A_{802}^{1cm} \simeq 30$) were stored at −70 °C in TL buffer (10 mM Tris, pH 8.0, and 0.025% LDAO).

Phospholipids. α-L-Lecithin from soybeans was partially purified as described by Kagawa & Racker (1971) and kept in an inert atmosphere at −40 °C. This preparation contains mostly phosphatidylcholine and phosphatidylethanolamine with smaller amounts of cardiolipin, monophosphoinositides, and other phospholipids (Kagawa & Racker, 1966; O'Brien et al., 1977).

Preparation of the RC–Lipid Complex in Hexane. All steps were performed under dim green light. Phospholipids were dispersed in 0.1 M KCl–10 mM imidazole, pH 7, to a final concentration of 15 mg/mL. Oxygen was excluded from the solution and the atmosphere above it by flushing with nitrogen gas. About 5 nmol of the RC stock in 50 μL of TL buffer was added to each 1-mL sample of the lipid suspension in screw cap culture tubes (13 × 100 mm). The suspension was mixed, flushed with N_2, and incubated at 25 °C for 30 min. Next, the mixture was sonicated for ~15 min at 25 °C in a 1700W bath sonicator operating at 25 kHz (Delta Sonics, Inc., Hawthorne, CA). The test tubes were held in preselected positions in the bath by a perforated board. These positions were selected by monitoring the acoustic field with a piezoelectric element (Vernitron Piezoelectric Division, Bedford, OH) fitted inside a vial and maximizing its output voltage. The suspension inside the test tubes was kept just below the level of the water in the bath. The effectiveness of the sonication was periodically monitored by measuring changes in the optical absorbance at 650 nm with a spectrophotometer (Spectronic-20, Bausch & Lomb, Inc., Rochester, NY). The turbid suspension became optically clear during sonication, and the process was considered to be complete when the optical absorbance decreased to a constant value of ~0.2. Fifty microliters of 1 M $MgCl_2$ and 1 mL of hexane were then added to each test tube. The test tubes were shaken with a vortex mixer for 5 min at 4 °C, and the two phases were separated by centrifugation for 5 min at 1700*g* and 25 °C. The upper (hexane) phase was retained.

Analytical Methods. Phospholipid phosphorus was analyzed according to Ames & Dubin (1960). Magnesium concentration was determined by atomic absorption spectroscopy (Model AA-5 spectrophotometer, Varian Techtron PTY, Melbourne, Australia). For determination of the magnesium content of hexane extracts, the solvent was first evaporated and the residue solubilized in 1% LDAO, 50 mM EDTA, and 10 mM Tris, pH 8. Radioactivity was measured with a liquid scintillation counter (LS-100, Beckman, Fullerton, CA) using a detergent-based scintillation liquid (Scintisol, Isolab, Inc., Akron, OH).

The amount of water in the hexane phase was determined by two independent methods: tritiated water and NMR spectroscopy. In the first method, tritiated water was added to the original lipid suspension and the radioactivity in the hexane phase was measured after phase separation. NMR spectral measurements were carried out by adding to the hexane extract 1.2% benzene as a standard and computing the areas under the benzene and water peaks. The water peak was identified by using D_2O instead of water in the aqueous phase.

(*1*) *Optical Spectroscopy.* Spectra were measured at 25 °C with a Cary 14R spectrophotometer (Applied Physics Corp., Monrovia, CA). Actinic illumination for steady-state and slow (~1 s) optical absorbance changes was provided by the IR-2 mode of the Cary spectrophotometer or by a slide projector equipped with a 500-W lamp. The light intensities at the position of the cuvette were 0.6 and 0.4 W/cm^2, respectively. For fast (~1 ms) absorbance changes, a modified Cary 14 spectrophotometer connected to a storage oscilloscope was used (Nicolet 1090 AR, Nicolet Instrument Corp., Madison, WI). The actinic light in this case was provided by single saturating flashes produced by a flash unit with an input level setting of 1200 W (Norman P 2000 D, Norman Enterprises, Inc., Burbank, CA).

(*2*) *EPR Spectroscopy.* The EPR spectrophotometer, light source, and filters described by McElroy et al. (1974) were used in this work. The EPR cells were made from a 1-mm thick quartz frame (25 × 10 mm) to which quartz cover slips were attached with silicone grease. After filling the cells, they were immediately frozen in liquid nitrogen (in the dark).

(*3*) *NMR Spectroscopy.* NMR spectra of samples in 5 × 180 mm glass tubes were obtained with a Varian HR-220 spectrometer (Varian Instrument Div., Palo Alto, CA) equipped with a Nicolet Technologies Fourier transform accessory (Nicolet Instrument Corp., Madison, WI). Spectra with a 2500-Hz spectral width were acquired by using a 10-μs pulse width (90° pulse equals 50 μs) at 31.6-s intervals. A 5-Hz line broadening was applied before Fourier transformation, 8000 data points were acquired, and 4 acquisitions of each spectrum were averaged. The experiments were done at 25 °C.

Results

(*A*) *Preparation of RC–Phospholipid Complex in Hexane.* The extent of transfer of RCs from water into hexane and the spectral integrity of the protein were monitored by optical absorbance measurements. The optical spectrum of purified RCs in aqueous detergent solution is shown in Figure 1A. The distinct features of this spectrum are the three peaks in the infrared region at 760, 800, and 865 nm. Figure 1B illustrates the results of an attempt to solubilize purified RCs in hexane by sonication in the absence of phospholipids. The optical spectrum of this extract shows a single absorbance peak at 760 nm which indicates the extraction of the RC pigments by the organic solvent and denaturation of the protein. Figure 1C shows the optical spectrum of an RC–lipid complex in hexane which was obtained by following the procedure outlined under Materials and Methods. This spectrum is similar to that of purified RCs in water (Figure 1A).

The optimization of the preparation of the RC–lipid complex in hexane was carried out with respect to two criteria: (a) the yield of transfer of RCs into the hexane phase and (b) the

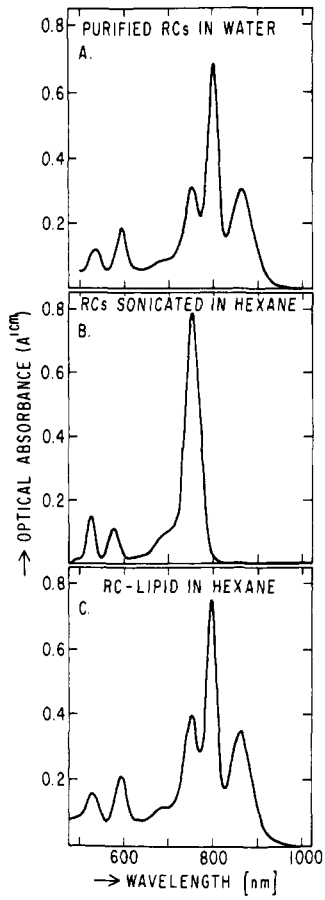

FIGURE 1: Optical spectra of RCs in water and in hexane. (A) Spectrum of RCs in water. Purified RCs were suspended in TL buffer. (B) Spectrum of RCs sonicated in hexane. 5 nmol of RCs in 50 μL of TL buffer was sonicated in 1 mL of hexane for 25 min. The spectrum was taken after centrifugation at 1700*g* for 5 min. (C) Spectrum of RC–lipid complex in hexane, prepared as described under Materials and Methods.

preservation of the native spectral characteristics of RCs. For the yield, the concentration of RCs in the hexane phase was determined optically by using the known extinction coefficient at 802 nm. This peak was used in preference to the 865-nm peak since the amplitude of the latter depends more critically on the oxidation state of RCs. For the determination of the native spectral characteristics, we adopted as an operational criterion the absorbance ratio A_{865}/A_{760}. This ratio equals approximately unity in purified native RCs (Figure 1A) and approaches zero in denatured RCs from which the pigments are extracted by the organic solvent (Figure 1B).

The optimization procedure was carried out by varying one parameter at a time at each stage of the extraction procedure (see Materials and Methods), initially assigning arbitrary values to the other parameters. At each successive stage, the optimum values of previously investigated parameters were used. After the completion of this series of experiments, the whole process was repeated until a set of measurements was obtained in which the effects of varying each parameter were tested under optimal conditions of all other parameters. The data presented below are the final results of these experiments.

(*1*) *Phospholipid Concentration.* In the absence of phospholipids, the transfer yield of RCs into hexane was negligible (Figure 2A). Increasing concentrations of lipid in the aqueous phase increased the yield as measured by A_{800} in the hexane phase. The lipid requirement reached saturation at ~15 mg/mL. The absorbance ratio A_{865}/A_{760} increased with increasing lipid concentrations (Figure 2B), reaching a steady

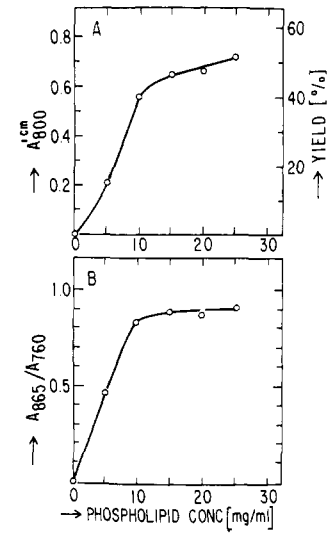

FIGURE 2: Effects of soybean phospholipid concentration in the aqueous suspension on the yield of extraction as measured by the optical absorbance at 800 nm (A) and on the native spectral characteristics as measured by the absorbance ratio A_{865}/A_{760} in the hexane phase (B). A 100% yield corresponds to a complete transfer of RCs from the aqueous phase to the hexane phase.

level of 0.9 at 10 mg of phospholipid per mL. At this phospholipid concentration, the spectral characteristics of the RCs were preserved. The transfer of RCs into hexane was also accomplished by using a *single* phospholipid species, dioleyllecithin, instead of the soybean phospholipids. However, the extraction yield in this case was low (25%). No further attempts were made at this stage to optimize the extraction of RCs with dioleyllecithin.

(*2*) *Preincubation with Lipid and Detergent Dilution.* Purified RCs are routinely stored in TL buffer in which the detergent is just above the critical micelle concentration (Tokiwa & Ohki, 1968). Lowering the concentration of LDAO below this point resulted in gradual precipitation of RCs in the absence of lipid. However, in the presence of lipid this increased the extraction yield. A 20-fold dilution of the RC stock with the lipid suspension (which brought the final LDAO concentration to 0.00125%) resulted in a high transfer yield of the RC–lipid complex into hexane. Further reduction of detergent concentration with Bio-Beads resulted in partial precipitation of RCs with a concomitant reduction in yield.

RCs were incubated with the lipid at 25 °C before the sonication step in order to maximize the effectiveness of the detergent dilution. An incubation period of 30 min or more increased the overall yield by ~20%.

(*3*) *Sonication.* Figure 3A illustrates the effect of sonication time of the RC–lipid mixture on the transfer yield of RCs into hexane. The absorbance at 800 nm of the hexane phase was negligible without sonication and increased with time, reaching a maximum after 10–20 min of sonication. The spectral integrity of the protein as measured by the ratio A_{865}/A_{760} also improved with sonication time, reaching a maximum close to unity after ~10 min (Figure 3B). However, longer sonication periods decreased both parameters, indicating that the protein gradually denatured under these conditions.

The turbidity of the aqueous suspension of phospholipids and RCs was monitored during sonication by measuring the optical absorbance at 650 nm (where the optical spectrum of RCs shows a minimum). Figure 3C shows that this absorbance decreased during sonication, reaching a constant value after ~10 min. Comparison of parts A and C of Figure 3 establishes a correlation between the decrease in A_{650} during

Table I: Composition of Hexane Extract and Aqueous Phase after Centrifugation[a]

fraction	concn					molar ratio		
	RCs (μM)	soybean lipids (mM)	LDAO (mM)	Mg (mM)	H_2O (M)	lipid/protein	LDAO/protein	lipid/LDAO
hexane phase	2.6	12.5	0.85	2.7	0.5	4800	326	15
aqueous phase	3.1	2.0	0.12	45	55.5	650	38	17

[a] Preparation of the samples and analytical methods were as described under Materials and Methods. See the text for further details.

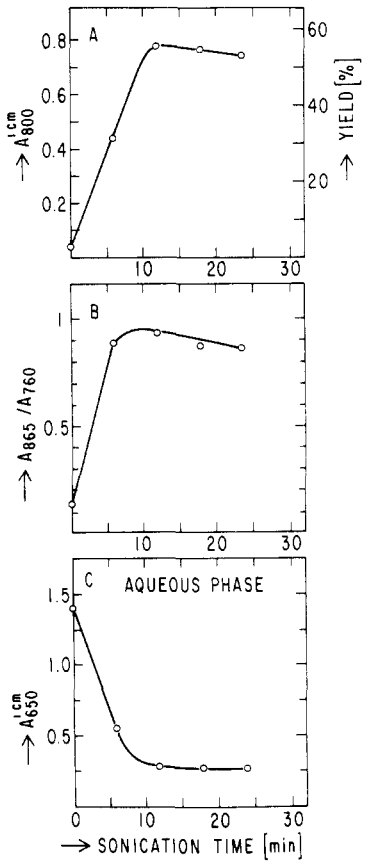

FIGURE 3: Effect of sonication time on the extraction yield (A) and on the native spectral characteristics (B) of RC–lipid complex in hexane. Light scattering of the aqueous suspension before extraction was measured by the absorbance A_{650} (C).

sonication and the increase in A_{800} of the hexane phase. Ineffective sonication was found to be the cause of occasional low yields; consequently, the monitoring of A_{650} was important to achieve high reproducibility.

(*4*) *Addition of Cations.* Addition of cations was found to be necessary for the transfer of the RC–lipid complex into the organic solvent. The transfer yield reached saturation at ~50 mM $MgCl_2$. The increase in yield was accompanied by a similar increase in the absorbance ratio A_{865}/A_{760}. Calcium could replace magnesium with equal effectiveness.

(*5*) *Organic Solvents.* Although hexane was the solvent routinely used, we also tried to extract the RC–lipid complex into other organic solvents. Of those tested, only alkanes were found to preserve the spectral integrity of RCs, while other solvents extracted the pigments from RCs and denatured the protein. We tested the denaturing potency of the different solvents by adding each one at 25% in volume to a stable hexane extract of the RC–lipid complex and measuring the absorbance ratio A_{865}/A_{760} of the mixture after 1 min and after 10 min of incubation at 25 °C. The solvents tested, listed in the order of increasing denaturing effects, were methylcyclohexane, benzene, carbon tetrachloride, diethyl ether, chloroform, butanol, methylene chloride, acetone, and ethanol. The presence of 1% ethanol (or methanol) in the hexane extract was sufficient to denature the protein. All alkanes tested (pentane, hexane, heptane, and octane) were equally effective as far as the yield and the preservation of the spectral integrity of the RCs were concerned.

(*B*) *Properties of RC–Phospholipid Complex.* (*1*) *Composition of Hexane Extract.* Table I presents the composition of a typical hexane extract, together with the composition of the aqueous phase obtained after phase separation. All the experimental variables discussed in the preceding sections were kept at their optimum values. About 45% of the RCs and almost 90% of the phospholipids that were initially present in the aqueous phase were transferred into the hexane phase. The phospholipid/protein molar ratio in the hexane phase was 4800 in this preparation and varied between 4500 and 6500 in other experiments. The RCs were solubilized and purified from chromatophores by using radiolabeled [^{14}C]LDAO in order to measure the amount of detergent. Table I shows that similar to phospholipids, nearly 90% of the detergent initially present in the aqueous phase was transferred into the organic phase. The mole ratio of detergent to protein in the hexane phase was usually between 300 and 400. The amount of magnesium transferred into the organic solvent was 2 to 3 μmol out of 50 μmol of $MgCl_2$ initially added.

By use of ^3H_2O, the water content of the hexane extract containing 2.6 μM RCs was found to be 0.50 ± 0.05 M. A maximum correction for possible exchange of tritium with other components in the hexane phase accounted for less than 5% of the label found in the extract.[2] Therefore, at least 95% of the tritium was associated with water. The water content was also determined by NMR spectroscopy (Figure 4). This method gave, within experimental error, the same result as the first one, i.e., 0.52 ± 0.05 M water in a hexane extract that contained 2.6 μM RCs (see Table I).

(*2*) *Size of Complex.* The maximum size of the complex was estimated by passing the hexane extract through Nuclepore filters of varying sizes and determining the concentration of RCs in the filtrate. Figure 5A shows the results of these experiments. RC–lipid complexes passed freely through filters with pore sizes larger than or equal to 0.1 μm and were retained to varying degrees by filters with smaller pore sizes. Filters with pore sizes smaller than 0.1 μm tended to clog during the experiments so that the concentration of RCs in the filtrate gradually decreased. This effect was taken into account in the analysis, collecting and assaying small fractions of the filtrate (20–50 μL) and extrapolating the concentration to zero volume. The size of the RC–lipid complexes, as obtained from the results shown in Figure 5A, showed a broad distribution between 0.015 and 0.08 μm, with a median size of 0.055 μm. The same method was utilized to measure the size of the RC–detergent complexes in TL buffer (Figure 5B). Their size ranged between 0.015 and 0.05 μm, with a median

[2] The lipid concentration in the hexane extract was 12.5 mM (Table I). By the assumption that 1 to 2 protons per lipid molecule are exchangeable with tritium, the correction amounts to 2.5–5%. Contributions from the protein and detergent are negligible (<1%).

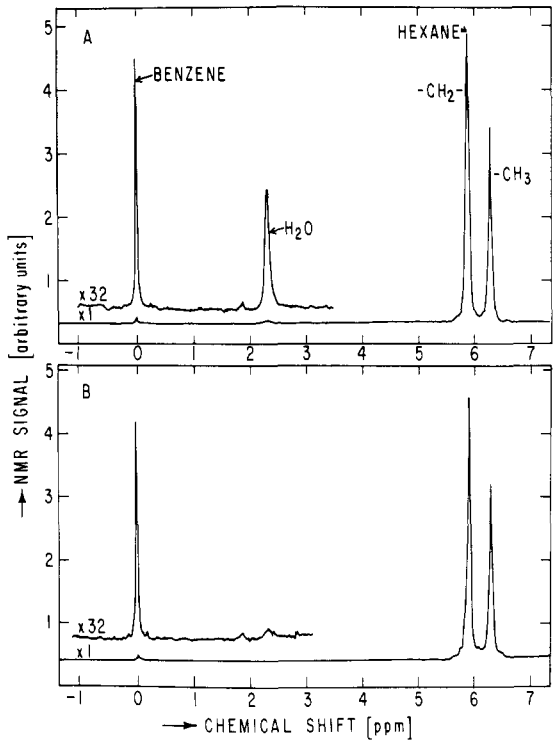

FIGURE 4: NMR spectra of hexane extracts prepared with water (A) and with 87% D_2O (B). Benzene (1.2% v/v) was added to each sample as a standard. The preparation of hexane extracts of the RC–lipid complex and details of the measurements are given under Materials and Methods. The final RC concentration was 2.6 μM. Note the two hexane peaks on the right side and the benzene peak on the left side of each frame. The water peak seen in (A) (in the middle) is greatly diminished in (B).

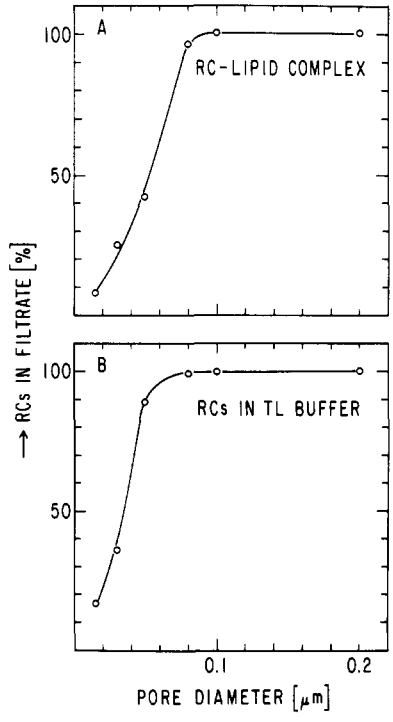

FIGURE 5: Size determination of RC-containing particles by ultrafiltration. RC–lipid complex in hexane (A) or purified RCs in TL buffer (B) were passed through Nuclepore filters of different pore sizes. The concentration of RCs in the filtrates was measured by their absorbance at 800 nm. Samples were forced through the filters by gas pressure (up to 6 kg/cm^2). See the text for further details.

size of 0.035 μm. This value is larger than that measured by gel filtration or equilibrium sedimentation (Reiss-Husson &

Table II: Composition of RC-Lipid Complex[a]

	RCs		lipid		water	
sample	μM	%	mM	%	M	%
hexane extract	2.4	100	12.5	100	0.54	100
pellet	2.4	~100	7.7	62	0.43	80
supernatant	<0.03	<1	4.7	38	0.11	20

[a] Distribution of phospholipid and water between the RC-lipid complex (pellet) and solvent (supernatant). Hexane extract of the RC-lipid complex (see Table I for detailed composition) was centrifuged at 110000g for 1 h. The concentrations of RCs, phospholipids, and tritium-labeled water were determined in both the pellet and the supernatant.

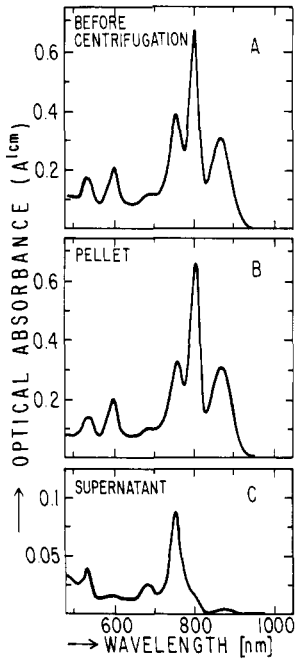

FIGURE 6: Optical spectra of RC–lipid complex in hexane before (A) and after (B) centrifugation. The hexane extract was centrifuged at 110000g for 1 h at 4 °C. The pellet was resuspended in hexane at a volume equal to the original one. The spectrum of the supernatant is shown in (C). Note the change in scale of the ordinate.

Jolchine, 1972). This difference may be attributed to either partial aggregation of RCs due to low LDAO concentration or partial occlusion of the filters as mentioned before.

(3) Composition of Complex. The RC–lipid complex was sedimented at 110000g for 1 h. By measurement of the lipid and water content of the pellet, the amounts actually associated in a complex with the RCs were determined. As Table II shows, about 60% of the lipid and 80% of the water present in the hexane extract sedimented with the RCs while the rest remained in solution. The detergent was approximately equally distributed between the pellet and the supernatant (data not shown).

After centrifugation and resuspension of the pellet in hexane, a decrease in absorbance of the 760- and 530-nm peaks was observed (parts A and B of Figure 6). Concomitantly, the supernatant showed an increase in absorbance at these peaks (Figure 6C). These spectral changes indicate that during centrifugation *free* bacteriopheophytin (and some bacteriochlorophyll) was removed, thereby effecting a further purification of the RC–lipid complex. The spectrum of the resuspended pellet (Figure 6B) closely matches that of purified RCs (Figure 1A).

The amount of phospholipids associated in a complex with the RCs could be reduced by repeated centrifugation and

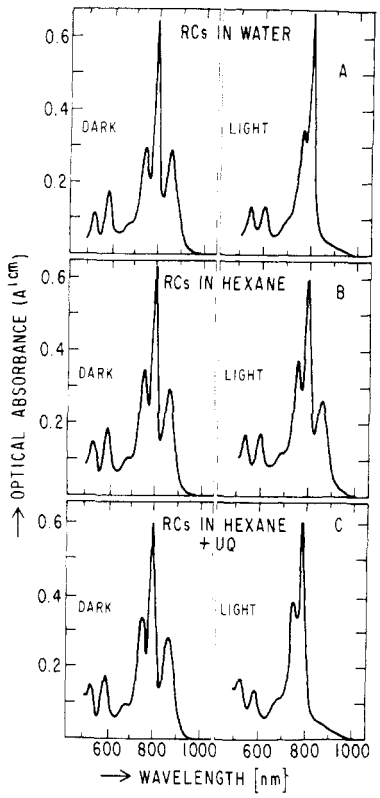

FIGURE 7: Optical spectra of RCs and RC–lipid complex in the dark (left frames) and when illuminated with actinic illumination (right frames). (A) Spectra of RCs in TL buffer. (B) Spectra of RC–lipid complex in hexane. (C) Same as (B) after addition of 0.3 mM Q-10. The quinone was incubated with the hexane phase for 15 min at 25 °C before the assay. Actinic illumination was provided by the IR-2 mode of the Cary 14 spectrophotometer.

resuspension. Prior to centrifugation, the hexane extract was diluted fivefold in hexane and stirred for 2 h. In a typical experiment, the lipid/protein ratio was reduced from 6000 to 700 in the first centrifugation and to 170 after the third centrifugation. The lipid/LDAO ratio did not change significantly during this procedure.

(*4*) *Stability of Complex.* The RC–lipid complex is remarkably stable in hexane. It could be kept for several weeks at 4 °C with negligible changes in its spectral characteristics or its activity (to be described later).

The stability and solubility of the complex were reduced when the lipid content was decreased. RC–lipid complexes in hexane with lipid/protein ratios lower than 500 tended to aggregate, and a considerable fraction of the RCs sedimented at 2000*g*. After several days at 4 °C, *all* of the RC–lipid complexes settled down. Samples with low lipid content were also more susceptible to damage by the organic solvent, and comparatively larger amounts of extracted pigments appeared in the supernatant when the complex was pelleted.

(*C*) *Photoactivity of RC–Phospholipid Complex.* (*1*) *Primary Photoactivity: Optical Spectroscopy.* The primary photochemical reaction carried out by RCs is accompanied by light-induced absorbance changes. The most prominent change is the reversible bleaching of the peak at 865 nm, as shown in Figure 7A.

The hexane extract exhibited optical absorbance characteristics in the dark that were similar to those of purified RCs. In contrast, the light-induced absorbance changes amounted to only ~5% of the expected values (Figure 7B). However, the photoactivity could be almost fully restored by adding excess exogenous ubiquinone (Q-10; Figure 7C). RCs are known to contain 2 equiv of Q-10 which are believed to act as primary and secondary acceptors [see Feher & Okamura (1978) for a discussion]. Evidently, the quinones were extracted by the organic solvent, resulting in loss of activity.

We studied the restoration of activity by correlating photochemical activity with the amount of Q-10 added. With the addition of 0.5 mM Q-10, ~75% of the maximum activity was recovered. The quinone had to be preincubated in the hexane fraction for at least 10 min before the assay in order to obtain this activity.

Sedimentation of the complex and resuspension in hexane did not affect its photoactivity. The activity was preserved even after three consecutive washings in hexane. Similar photoactivities were also exhibited by RC–lipid complexes that were formed in pentane or heptane.

Resuspension of RCs in Water. The photochemical activity of the RC–lipid complex could be restored *without* the addition of quinone by resuspension in water. This was accomplished by evaporating the hexane and resuspending the RCs in 1% LDAO and 10 mM Tris, pH 8. The resultant dispersion showed the characteristic optical spectrum of native RCs as well as complete photoactivity. Recombination of the RCs with the quinone that was originally associated with them was evidently achieved once the solvent was evaporated.

(*2*) *Primary Photoactivity: EPR Spectroscopy.* Active RCs display two light-induced EPR signals: a narrow signal at $g = 2.0026$ associated with the oxidized primary electron donor, D (a specialized bacteriochlorophyll dimer), and a broad signal at $g = 1.8$ associated with the reduced primary acceptor, A (a ferroquinone complex) (Feher & Okamura, 1978).

The time constant of the dark back reaction, τ_D, is believed to be a sensitive measure of the native conformation of RCs (McElroy et al., 1974). Similarly, the shape of the broad EPR signal at $g = 1.8$ reflects the conformation of the iron–quinone complex (Okamura et al., 1975). We therefore determined and compared both of these parameters for RCs in TL buffer with those obtained for the RC–lipid complex in hexane. Figure 8 shows that they were approximately the same for both preparations. A closer analysis revealed small changes (10–20%) in line shape and width of the broad signal (parts A and B of Figure 8), as well as in the kinetic parameters (parts C and D of Figure 8). RCs in hexane had a somewhat longer τ_D (~40 ms instead of 30 ms); in addition, the decay had a 10–20% component with a significantly longer (~300 ms) time constant.

The relative amplitude of the broad signals shows that the hexane preparation to which exogenous Q-10 was added was ~80% active at low temperatures. When Q-10 was not added to the hexane extract, the amplitude of the EPR signal decreased to 10% of its maximum value. The broad line width of the peak at $g = 1.8$ has been attributed to the interaction of the magnetic moment of the Fe^{2+} with the electron spin of $Q\text{-}10^-$. The presence of this resonance in the hexane extract, therefore, shows that the iron had not been extracted from the RCs.

(*3*) *Exogenous Electron Carriers.* Removal of ubiquinone from purified RCs abolishes their photochemical activity. The activity was restored by the addition of either Q-10 or one of several other quinones (Cogdell et al., 1974; Okamura et al., 1975). Figure 9 shows that several hexane-soluble quinones could substitute for Q-10 in supporting the light-induced bleaching at 865 nm (i.e., production of D^+) of the RC–lipid complex in hexane. The light-dependent changes in the optical spectrum of the RCs were similar to those obtained with Q-10 (Figure 7).

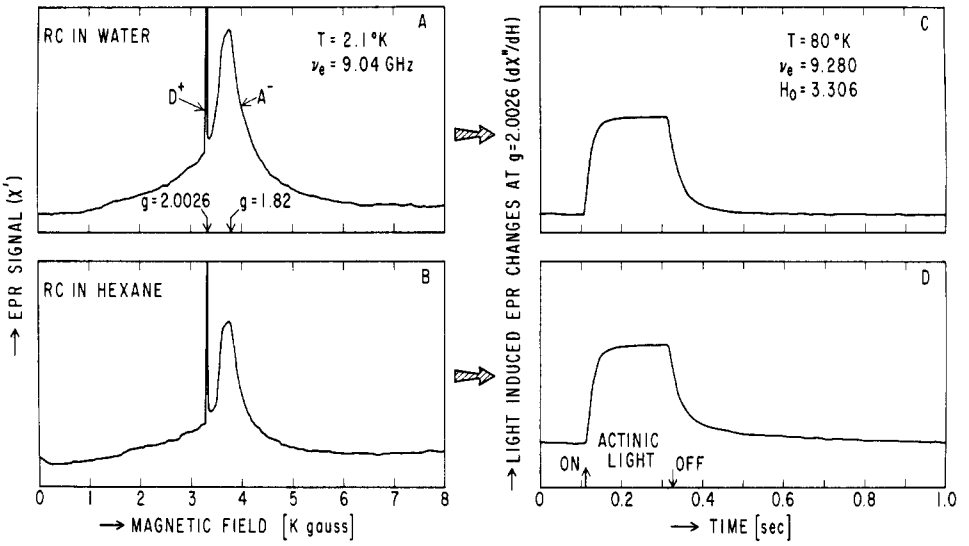

FIGURE 8: Comparison of low-temperature EPR characteristics of RCs in water and in hexane. The hexane fraction was concentrated 10-fold to a final absorbance of $A_{800}^{1cm} = 6$, the same as for RCs in water. The exogenous Q-10 concentration in the hexane fraction was increased to maintain a quinone/protein mole ratio of ~2500. The optical path length of EPR cells was 1 mm. EPR spectra at 2.1 K were obtained with light modulation (A and B). Kinetic data at 80 K were obtained with magnetic field modulation (C and D).

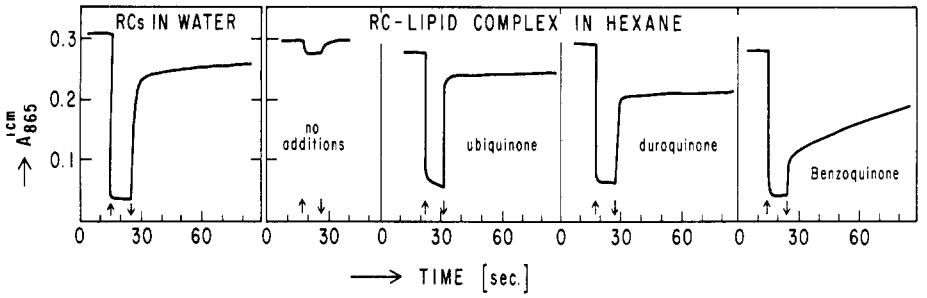

FIGURE 9: Time course of light-induced absorbance changes at 865 nm of RCs in aqueous detergent solution and of the RC–lipid complex in hexane in the absence and presence of different exogenous electron acceptors at 1 mM concentration. Actinic illumination was provided at 90° to the measuring beam by a 500-W Leitz projector. The light was filtered through 5 cm of water; its intensity at the position of the cuvette was 0.4 W/cm^2. The duration of the actinic light pulse is indicated by arrows (↑ = on; ↓ = off).

As seen in Figure 9, a slow component (τ_D > 1 min) appeared in the dark recovery of the 865-nm change of RCs both in water and in hexane when prolonged (~10 s) actinic illumination was used. This slow component appeared also under anaerobic conditions and is probably due to transfer of electrons to an unidentified secondary acceptor which slows down the charge recombination at the level of the primary reactants. The rereduction of D^+ is accelerated when the reduced exogenous acceptor is also a potent electron donor (e.g., DAD, Figure 10A; discussed in the next section).

Reaction with Diaminodurene (DAD). DAD has been shown to stimulate cyclic photophosphorylation in chromatophores (Gromet-Elhanan, 1970) and also to act as a secondary electron donor with isolated RCs (Vermeglio, 1977; Wraight, 1977). Depending on its oxidation state, we found that it reacted with the RC–lipid complex in hexane, both as an electron donor and as an electron acceptor. Oxidized DAD, in the absence of ubiquinone, acted as a primary acceptor in supporting the bleaching at 865 nm (Figure 10A). Reduced DAD, in the presence of ubiquinone, rereduced D^+, as expected, thereby diminishing the bleaching of the 865-nm peak (data not shown). Thus, in the presence of a mixture of oxidized and reduced DAD, the initial bleaching at 865 nm was followed by an almost complete rereduction. At this point, under steady-state illumination the rate of oxidation of D was equal to the rate of its reduction. On turning the actinic light off, the fully reduced state of D was obtained. Purified RCs in water (which contained Q-10) reacted with reduced DAD

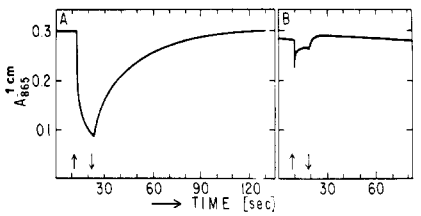

FIGURE 10: Time course of light-induced absorbance changes at 865 nm of RC–lipid complex in hexane in the presence of 0.1 mM oxidized DAD (A) and 0.1 mM oxidized DAD + 0.1 mM reduced DAD (B). Other details are as in Figure 9.

in a similar manner to that shown in Figure 10B, with DAD substituting for the native electron donor, cytochrome *c*.

Discussion

The reaction center of photosynthetic bacteria is a membrane protein that is insoluble in water. Its solubilization is achieved by substituting the membrane phospholipids of its native environment with detergent molecules. Despite its hydrophobic character, the RC (when associated with detergent) is insoluble in organic solvents. However, when RCs are associated with phospholipids in the presence of suitable counterions, they are transferable into alkanes (Figure 2A); this is in agreement with previous results obtained with other membrane proteins (Gitler & Montal, 1972; Montal & Korenbrot, 1973; Montal, 1974; Darszon et al., 1978). Furthermore, the lipids perform the important function of pro-

tecting the RCs against the denaturing effects of the solvent (Figure 2B).

The RC–lipid complex in hexane is photochemically active as shown by the preservation of the characteristic light-induced optical absorbance changes. The primary photoreaction was performed in the organic solvent provided that the complex was supplemented with Q-10 or several other hexane-soluble quinones. The similarities of the EPR characteristics of RCs in detergent and those of the RC–lipid complex in hexane establish that native conformation of RCs is maintained in the organic solvent.

The transfer of the RC–lipid complex into the organic solvent most likely proceeds by phase inversion; the hydrophilic parts of the lipid and protein which previously interacted with the bulk aqueous phase are now enclosed in a polar core surrounded by the phospholipid and detergent hydrocarbon chains, which in turn are in contact with the bulk hydrocarbon phase. This simple model allows us to estimate the dimensions of the complex from its composition (Table II). The average molecular weight of a particle containing one RC plus lipid, water, and detergent associated with it is $\sim 6 \times 10^6$. The water content, accounting for about half of the total weight, would fit in a central droplet having a diameter of 0.022 μm. Most of the lipid present would fit in a single layer surrounding the surface of this particle, increasing its size to $\sim$0.027 μm. The observed sizes of the majority of RC-containing particles in the hexane extract were between 0.015 and 0.085 μm in diameter (Figure 5A). This large distribution in particle size is probably due to variations in water and lipid content. Furthermore, the larger particles may contain more than one RC.

In conclusion, we have shown that the reaction center can be extracted into organic solvents while preserving photochemical activity. The RC–lipid complex reacted with exogenous electron carriers dissolved in the bulk hexane phase, thus performing biochemical reactions in an organic solvent. The photochemical activity of RCs was also retained after solvent removal and rehydration, thus fulfilling the requirements for the successful reconstitution of the protein in functional bilayers. Such reconstitutions have recently been accomplished with planar bilayers (Schönfeld et al., 1979b) and large vesicles (Darszon et al., 1980).

Acknowledgments

We express our gratitude to A. Darszon for helpful discussions and advice, to M. Kendall-Tobias and A. R. Crofts for sharing their results with us, to E. Abresch for technical assistance and especially for preparing the reaction centers, to R. Isaacson for performing the EPR measurements, and to J. Wright for performing the NMR experiments.

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