Journal of Bioenergetics and Biomembranes, Vol. 19, No. 3, 1987

Reaction Centers from *Rhodopseudomonas sphaeroides* in Reconstituted Phospholipid Vesicles. I. Structural Studies[1]

Klaas J. Hellingwerf[2,3,4]

Received July 17, 1986; revised October 20, 1986

Abstract

Reaction centers (RCs) from *Rhodopseudomonas sphaeroides* were reconstituted into asolectin vesicles by cosonication. Equilibrium centrifugation on sucrose gradients showed that the vesicles were homogeneous in density (i.e., lipid-to-protein ratio) when reconstituted at a molar lipid-to-protein ratio between 500 to 1000. At lower ratios, a considerable fraction of RCs was not incorporated into closed vesicles, while at higher ratios, an increasing population of liposomes was protein-free. The average vesicle size decreased with increasing lipid-to-protein ratio, exhibiting considerable size heterogeneity within a sample. The average diameter of the largest and smallest population of vesicles, reconstituted at a molar lipid-to-protein ratio of 560, was 1200 and 400 nm, respectively. The orientation of reconstituted RCs with respect to the plane of the membrane was determined from the flash-induced rereduction kinetics of the special-pair bacteriochlorophyll dimer in the presence of reduced cytochrome *c*. The predominant orientation of RCs was such that the cytochrome *c* binding sites faced the external medium. The net orientation of RCs in reconstituted vesicles decreased with vesicle size and was strongly influenced by the ionic strength during reconstitution.

Key Words: Reaction centers; *Rhodopseudomonas sphaeroides*; reconstitution; proteoliposomes; asolectin; orientation; structure.

---

[1] Abbreviations: RC: reaction center; LDAO: lauryldimethylamine-*N*-oxide; UQ_0/UQ_0H_2: oxidized and reduced form of 2,3-dimethoxy-5-methyl-1,4-benzoquinone; CCCP: carbonyl-cyanide-trichloromethoxy phenylhydrazone; D/D^+: reduced and oxidized form of the primary electron donor of the reaction centers.

[2] Departments of Biology and Physics, University of California, San Diego, La Jolla, California 92093.

[3] Permanent address: Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. To whom correspondence should be addressed.

[4] During the course of this study K. J. H. was supported by a grant from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). This research was supported by grants from the National Institutes of Health (EY-02084) and from the Office of Naval Research (ONR-NOOO 14-79-C 0798) to M. Montal.

0145-479X/87/0600-0203$05.00/0

Introduction

Biochemical and biophysical studies of reaction centers (RCs) from purple nonsulfur bacteria have unraveled extensive information on the structure and the primary electron transfer events proceeding in this energy-transducing machinery of photosynthetic bacteria (Okamura *et al.*, 1982; Pachence *et al.*, 1983). RCs are membrane proteins that span the bacterial plasma membrane, with an asymmetric distribution of protein mass and functional groups (Pachence *et al.*, 1983; Valkirs and Feher, 1982). The primary reaction catalyzed by the RCs is the transduction of light energy into an electrical potential and redox energy gradient across the membrane (the "charge-separation reaction," cf. Likhtenstein *et al.*, 1982). A cytochrome b/c_1 complex subsequently transforms these gradients into a protonmotive force via the active extrusion of protons coupled to the electron transfer reactions (Mitchell, 1968; Dutton *et al.*, 1982). The protonmotive force allows a bacterium to perform a large number of energy-consuming processes (Konings *et al.*, 1982; Hellingwerf and Konings, 1985).

Isolated RCs were reconstituted into model membranes of well-defined lipid composition both in vesicular and planar configuration (Crofts *et al.*, 1977; Pachence *et al.*, 1979; Schönfeld *et al.*, 1979). Reconstituted RC vesicles, when supplemented with redox mediators, translocate protons upon illumination (Crofts *et al.*, 1977; Darszon *et al.*, 1980). The reconstituted proton-translocating RC vesicles are a valuable system to explore the influence of a protonmotive force on the primary electron transfer reactions and a starting point for functional reconstitution studies using additional complexes that transduce chemiosmotic energy. These mechanistic investigations require a detailed characterization of the structure of the reconstituted vesicles. Here, we report such a study. A preliminary account of this work was presented elsewhere (Hellingwerf, 1984).

Experimental

Materials

Asolectin was purified from L-α-lecithin from soybean according to Kagawa and Racker (1971); it was stored in an inert atmosphere at −40°C. Horse heart cytochrome *c*, L-α-lecithin from soybean, and valinomycin were from Sigma Chemicals, St. Louis, Missouri; 2,3-dimethoxy-5-methyl-1,4-benzoquinone (UQ_0) was from Trans World Chemicals, Washington, D.C.; LDAO from Onyx Chemical Co., Jersey City, New Jersey; Bio-Beads SM-2 from Bio-Rad Laboratories, Richmond, California; ^{14}C-LDAO (3.4 Ci/mol)

was from ICN Corp., Irvine, California; L-α-(Dipalmitoyl-1-^{14}C-)-phosphatidylcholine (80–120 ci/mol) was from New England Nuclear, Boston, Massachusetts; and carbonylcyanide-trichloromethoxyphenylhydrazone (CCCP) from Calbiochem, La Jolla, California. All other chemicals were reagent grade.

Preparations

Reaction Centers. RCs were isolated and purified from *Rps. sphaeroides* R-26 as described (Feher and Okamura, 1978). Aliquots (1 ml) of RCs (10 mg protein/ml or an absorbance at 800 nm (A_{800}) of 30) in 10 mM Tris, pH 8.0, plus 0.025% lauryldimethylamine-*N*-oxide (LDAO) were stored at −70°C until use. Such RCs are referred to as "LDAO-containing RCs." The purification of RCs and all subsequent manipulations were performed in the dark or in dim green light.

LDAO-depleted RCs were prepared from LDAO-containing RCs (routinely between 1 and 4 ml) by dilution with 7 volumes 200 mM Tricine buffer, pH 8.0, and addition of Bio-Beads SM-2 (Holloway, 1973) 10% (w/v). After 3 h at room temperature, the mixture was centrifuged (100,000 × g; 15 min) and the sedimented RC pellet was separated from the supernatant, containing the floating Bio-Beads, and washed with 200 mM KCl. The pellet was resuspended in 200 mM KCl (at an $A_{800} = 10$) and centrifuged, and the RC pellet was resuspended in 200 mM KCl at 10 mg protein/ml ($A_{800} = 30$). A homogeneous suspension of the LDAO-depleted RCs was obtained after vortexing (~30 s). The LDAO-depleted RCs were stored at 4°C and used within 2 to 3 weeks. This procedure routinely led to a loss of 10–30% RCs due to adsorption to the Bio-Beads. RCs isolated in (^{14}C-)LDAO were used to determine the extent of LDAO removal by this procedure: 66% of the LDAO originally bound to RCs was removed, corresponding to a reduction in the number of LDAO molecules bound per RCs from 250 to 85 mol/mol. The optical absorbance spectrum of the LDAO-depleted RCs exhibited a shift of the absorption maximum, λ_{max}, at 802–805 nm, whereas the λ_{max} at 865 nm broadened and shifted to 850 nm. Both changes reversed upon solubilization with detergents (LDAO, cholate, or Triton X-100) or upon reconstitution of the RCs with asolectin into vesicles. LDAO depletion did not change the ubiquinone content of the RCs (between 0.7 and 0.9 functional secondary quinone per RC).

Reconstitution of RC Vesicles by Sonication (Racker, 1973). A 0.2-ml portion of the stock suspension of RCs (either LDAO-depleted or LDAO-containing) was added to 0.2 ml asolectin (40 mg/ml in 200 mM KCl, suspended by vortexing for 30 min) in a 13 × 100 mm screw-cap culture tube (Schönfeld *et al.*, 1980). The mixture was vortexed for 1 min, flushed with argon for 20 s, and sonicated for 10 min in a 1700-W bath-type sonicator

(Delta Sonics, Inc., Hawthorne, California) operating at 25 KHz, at a position optimized for power output (Schönfeld *et al.*, 1980). The vesicles were stored at 4°C and usually prepared and used the same day. Considering the molecular weight of an average lipid molecule and an RC (700 and 95,000, respectively; Okamura *et al.*, 1982), this reconstitution procedure results in a lipid/RC molar ratio of 560. Hereafter, this procedure is referred to as the standard procedure. The pH during all reconstitutions was between 7.5 and 8.0, and the temperature was between 21 and 27°C.

Reconstitution of RC Vesicles by Cholate Dialysis (Kagawa and Racker, 1971). A 0.4-ml portion of RCs in LDAO was mixed with 0.4 ml 10% cholate (w/v) by vortexing for 30 s; 0.4 ml asolectin (see above) was subsequently added, and the vortexing repeated. Next, 4.3 ml of 500 mM Tricine, pH 8.0, was added, followed by 0.55 g Bio-Beads, and the mixture was shaken at room temperature for 3 h. Next, the Bio-Beads were floated by centrifugation and the remaining suspension was dialyzed 3 times against 2 liters 200 mM KCl at 4°C (each dialysis lasted at least 4 h). For proton-translocation measurements, 0.5 ml of the resulting vesicle suspension was added to 1.5 ml 200 mM KCl in the chamber for pH measurements. The RC content of the vesicles was measured spectrophotometrically at 800 nm.

Optical Spectroscopy. Spectra were measured at room temperature, using a Perkin-Elmer model 555 (Perkin-Elmer Corp., Norwalk, Connecticut), a Cary 14D or a Cary 17D spectrophotometer (Applied Physics Corp., Monrovia, California). Optical measurements of relatively slow changes in the oxidation/reduction state of cytochrome *c* and of the primary electron donor of the RCs (D), were carried out using the Cary 14D, with a time resolution of approximately 1 s. Actinic illumination of equal intensity and similar wavelength as in the proton-translocation measurements (Hellingwerf, 1987) was supplied from a Leitz-Wetzlar 500-W slide projector. Narrow-band Corning interference filters were used to shield the photomultiplier of the spectrophotometer from the actinic illumination.

Optical kinetic measurements were performed with a spectrophotometer of local design (Kleinfeld, D., and Feher, G., in preparation), modified from an earlier version (McElroy *et al.*, 1974) with a time resolution of 1 μs. The actinic flash was supplied by a Phase-R DL2100C pulsed dye laser, using Rhodamine 590 dissolved in pure methanol as the dye. Flashes typically had an energy of 200 MJ during 400 nsec. The monitoring beam originates from the monochromator of a Cary 14D spectrophotometer, run off a DC lamp supply to remove AC ripple from the beam. Samples were held in four-walled micropolystyrene cuvettes (Scientific Products, McGaw Park, Illinois) in a thermostated jacket, kept at 21.5°C. After passing through the sample, the monitoring beam was filtered by a second monochromator (Bausch and Lomb, Rochester, New York) to block scattered laser light, and detected by

a Hammamatsu R666S photomultiplier tube. The signal was amplified and filtered with local electronics and recorded on a Nicolet 1090 AR digital oscilloscope. Data were then transferred to a Z-80 microcomputer-based computer system of local design for storage, manipulation, and/or graphing. Typically, kinetic data were plotted as the time dependence of the flash-induced bleaching of RCs at 865 nm, or as the logarithm of the dark recovery.

Analysis of the Density of Reconstituted RC Vesicles. Linear sucrose-density gradients were formed with a Pharmacia three-channel peristaltic pump from between 0 to 20 to between 30 to 70% (w/w) sucrose in 200 mM KCl, 0.5 mM EDTA, pH 8.0, in 5 ml centrifuge tubes. Approximately 0.2 ml RC or vesicle suspension was applied on top of the gradients, which were then centrifuged at 216,000 × g in a Beckman Model L2-65B ultracentrifuge (using the SW65L titanium rotor) for at least 48 h at 20°C. After centrifugation the gradients were fractionated by puncturing the bottom of the tubes and draining the contents into a Gilson fraction collector. The refractive index (with a Bausch and Lomb abbe 3-L refractometer) and the absorbance at 300 and 800 nm of 0.2 ml fractions was measured; from each fraction, 15 μl aliquots were used for the measurement of refractive index and the rest was diluted with 0.5 ml 200 mM KCl for optical analysis. Occasionally, the optical absorption spectrum of a fraction was measured between 300 and 900 nm to discriminate between actual absorbance at 800 nm due to the presence of RCs and apparent absorbance, due to light scattering.

Analysis of the Size of Reconstituted RC Vesicles.

(A) Light-Scattering Measurements. Light-scattering from reconstituted vesicles was measured with a device in which light from the 488 nm line from a Model 95 argon ion laser (Lexel, Palo Alto, California) is scattered at 90° from the solution in Airfuge microcells (Beckman, Spinco Division, Palo Alto, California), which were inserted into a square 1 × 1 cm fluorescent cuvette filled with water. This furnishes a temperature-stabilized medium and rough refractive index matching to reduce the scattering from the Airfuge cell. Centrifugation of the cell offers a way to clarify the solutions and to separate the scattering particles according to size. The scattered light is detected by a photomultiplier (RCA 7265). Photon pulses are amplified and fed into a Model 1096 photon correlator (Langely Ford Instruments, Amherst, Massachusetts) for data accumulation and analysis. The correlation is fitted by a single exponential, using the baseline as measured by the delayed channels. The results fluctuate by approximately 20% due to the existence of a wide range of vesicle sizes in many of the preparations, which affects the baseline. Therefore, the results on light scattering of the reconstituted RC vesicles are used only to derive qualitative comparisons of the various preparations, and the weight-averaged diameter of the vesicles was expressed as the nearest hundred angstrom.

(B) Freeze-Fracture Electron Microscopy. Samples were fixed in 2% glutaraldehyde for 5 min, cryoprotected in 30% glycerol, and rapidly frozen in Freon 22, cooled by liquid nitrogen. The samples were fractured, unidirectionally shadowed with platinum at an angle of 30°, and carbon stabilized using a Balzers 300 freeze-etch device equipped with a turbomolecular pump and quartz crystal-thin film monitor. Stereo pairs of the freeze-fracture replicas were taken with the JEOL 100 CX electron microscope using the eucentric goniometer stage at -5 and $+5°$ for subsequent three-dimensional examination.

Orientation of RCs in Reconstituted Vesicles. The orientation of RCs, operationally assayed as the availability of their cytochrome *c* binding site on the outer or inner surface of the membrane in reconstituted vesicles, was measured with the instrument for kinetic optical absorbance measurements. With this assay, the time-dependent recovery of flash-generated oxidized primary electron donor of the RCs (D^+) is measured in the presence of reduced cytochrome *c* outside the vesicles (Fig. 1), essentially as described by Pachence *et al.*, (1979). Routinely, 50 μl reconstituted vesicles were mixed with 0.75 ml of 200 mM KCl in polystyrene microcells. Subsequently, cytochrome *c* and UQ_0 were added (at a final concentration of 17 and 500 μM, respectively). After equilibration in the dark, the bleaching of the RCs at

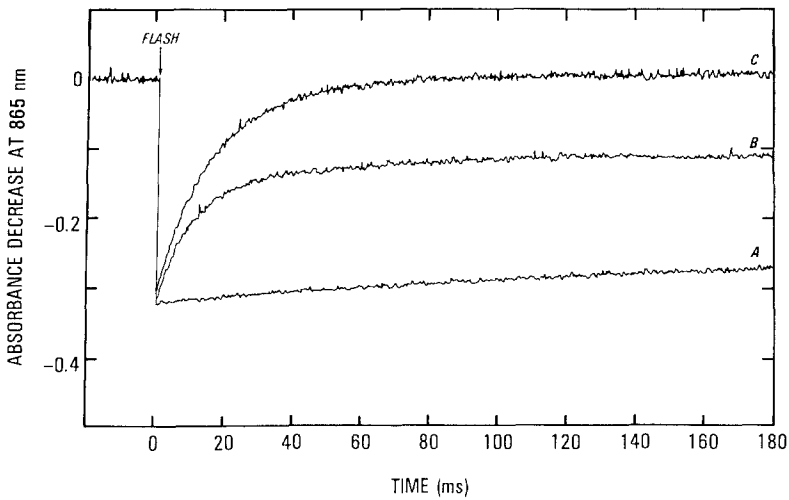

Fig. 1. Optical absorption assay for the orientation of reconstituted RCs. Vesicles were reconstituted from asolectin and LDAO-depleted RCs (molar ratio 560) with sonication according to the standard procedure (see Methods). In A, B and C, UQ_0, cytochrome *c*, and Triton X-100 were added successively, at a final concentration of 500 and 17 μM and 0.2%, respectively. At the arrow, the suspension was flashed. Calculation shows that 61% of the RCs have the *in vivo* orientation in this sample (compare Fig. 5).

865 nm by separate flashes (2 min apart) was recorded. The orientation of the RCs was calculated as the average from three separate absorption transients, as the contribution to the regeneration of the reduced primary electron donor of the RCs by reduced cytochrome *c* (Fig. 1B, the fast rereduction of D^+ that occurs only in RCs with their cytochrome *c* binding site facing the exterior of the reconstituted vesicles) relative to the rereduction from the primary or secondary quinone of the RCs (Fig. 1A, the slow phase, with a rate constant longer than 100 ms; see Fig. 1 for further details). The former RC's are referred to in this paper as RC's, having the *in vivo* orientation. Under the conditions used here [i.e., 200 mM KCl and cytochrome *c* (17 μM)], the rate constant for rereduction of D^+ is 77 s^{-1}. Upon addition of a detergent (either Triton X-100 or cholate, both at 0.1% (w/v) final concentration), a complete recovery of D^+ according to the fast rereduction process (rereduction via cytochrome *c*) was observed (Fig. 1C).

Proton Translocation Measurements. Proton translocation was measured as the change in pH of the external medium of a suspension of reconstituted RC vesicles, upon illumination. The instrument consisted of an O-I pH 2000 combination electrode (Owens-Illinois, Inc., Toledo, Ohio) connected to a Model 600B Keithley electrometer (Keithley Instruments., Inc., Cleveland, Ohio). The output signal from the electrometer was continuously recorded on a strip chart recorder (Hewlett-Packard, Model 7035B X–Y recorder). The flat-bottomed pH electrode (diameter 14 mm) was inserted sideways into a thermostated chamber, constructed by drilling a 16-mm-diameter hole in a Lucite block. The chamber had an adjustable working volume between 1.0 and 4.0 ml. It was mounted on a magnetic stirrer (Vanlab magnetic stirrer/hot plate, VWR Scientific Inc.,) for mechanical support and for mixing. It was thermostated at 30°C by means of a circulating water bath. The chamber was covered with a Teflon stopper or a cover glass and was flushed with water-saturated argon to obtain anaerobiosis. Light was supplied by an Ealing Model 22-0004 fiber light source (The Ealing Corp., South Natick, Massachusetts), equipped with a 150-W tungsten filament lamp. The light was passed through a 10-cm water filter which also focused the light on a mirror which in turn reflected the actinic light on the chamber. In addition, the light was filtered through a long-pass cut-off filter (50% transmission at 660 nm). With this arrangement, a light intensity of 40 mW/cm^2 (measured with an YSI-Kettering No. 65 radiometer) was obtained at the surface of the chamber. The pH of the suspension was read directly from the Keithley electrometer, after appropriate calibration of the system with regular pH calibration buffers between pH 4 and 10 (Scientific Products, McGaw Park, Illinois: Fisher Scientific Co., Fair Lawn, New York, and Mallinckrodt Inc., Paris, Kentucky). The pH changes in the chamber upon illumination were

calibrated with the addition of known quantities of oxalic acid. Due to the geometry of the chamber, high stirring speed ($\sim$ 50 Hz) is required for a rapid response of the electrode to pH changes; the response time was $\sim$ 1 s. The pH measurements were routinely performed after the successive addition to the chamber of 2.0 ml anaerobic 200 mM KCl, 50 μl 1 mM reduced cytochrome *c*, 5 μl 100 mM UQ_0 (partially reduced, see Materials), and $\sim$ 50 μl of a suspension of RC vesicles reconstituted by sonication (typically 0.1–0.5 mg RC protein) and 5 μl 1 mM valinomycin. Proton translocation measurements were performed in the presence of a saturating amount of valinomycin (i.e., 2.5 μM), which in 200 mM KCl collapses the transmembrane electrical potential thereby simplifying the interpretation of the measurements (Hellingwerf *et al.*, 1978). RC vesicles prepared by cholatedialysis were assayed in the same range of protein concentrations (Hellingwerf, 1987). When ethanolic solutions were added (cf. valinomycin, UQ_0, *o*-phenanthroline, etc.) the total ethanol content of the sample was kept below 1% (v/v). Preceding the actual measurements, the mixture was equilibrated for 20 min in the dark in the vessel for pH measurements to reduce the initial drift in the pH of the sample. Changes in pH as small as 10^{-4} pH unit ($\sim$ 1 nmol protons) are detected with this instrument. Measurements of the initial rate and extent of proton translocation on each sample were performed in replicate.

Miscellaneous. Radioactivity was assayed with liquid scintillation spectrometry, according to standard procedures. Light-induced proton translocation in the reconstituted vesicles was assayed as described in detail in the accompanying paper (Hellingwerf, 1987). These measurements were performed in the presence of 2.5 μM valinomycin (a saturating concentration) to simplify their interpretation (Hellingwerf *et al.*, 1978).

Results

Reconstituted RC Vesicles

Purified RCs are readily reconstituted into vesicles by cosonication with asolectin. Figure 2 shows that a sonication time of 10 min produces vesicles that exhibit light-dependent proton translocation to an extent that approaches optimum. Longer sonication periods ($\geqslant$ 20 min) promote the denaturation of RCs (as determined from the optical absorbance spectrum and the concomitant decrease in the extent of light-dependent proton translocation. For this reason, it was considered prudent to fix the sonication time at 10 min for all the subsequent reconstitutions to be characterized in terms of the structural parameters of the reconstituted vesicles. Figure 2 also shows that the net

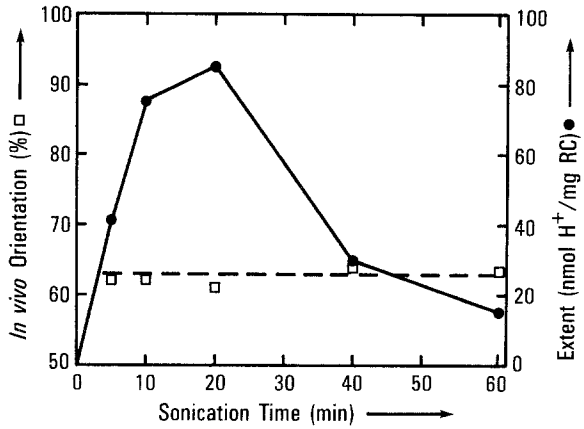

Fig. 2. Effect of time of exposure to ultrasonic irradiation during reconstitution on light-induced proton translocation and orientation of RCs in reconstituted vesicles. RC vesicles were reconstituted from asolectin and LDAO-depleted RCs (molar ratio 560), essentially as described under Methods, except that the sonication time varied between 5 and 60 min and the volume of the vesicle suspension was 0.5 ml at the start of the experiment. At each time point an aliquot of 70 μl was withdrawn for measurement of (i) the orientation of the RCs (ii) the extent of light-dependent proton translocation, (iii) the spectral characteristics of the reconstituted RCs, and (iv) light scattering of the vesicles. Following each withdrawal, the remaining vesicle suspension was reflushed with argon.

orientation of the RCs was virtually independent of the time of exposure of the vesicles to ultasonic irradiation.

Density of the Reconstituted RC Vesicles

Figure 3 shows a density-gradient analysis of RC vesicles reconstituted at a lipid/RC ratio of 280 and 1400, respectively. Upon centrifugation to equilibrium (Hellingwerf, 1979) it could be seen that all LDAO-depleted RCs were incorporated into vesicles when the lipid-to-protein ratio was 1400 (Fig. 3B). The same holds for the majority ($\geqslant 90\%$, see below) of the RCs reconstituted at a ratio of 280. Upon fractionation, a significant peak broadening occurs, as seen from visual inspection of the gradient before and after fractionation. This suggests that at both ratios a homogeneous population of vesicles is obtained with a narrow range of densities. The absorbance at 300 nm, dominated by the light scattering from the reconstituted vesicles, agrees with this view. Clearly the average density of the reconstituted vesicles is lowest at the highest lipid/RC ratio, and the shoulder on the low-density side of the peak in the 300 nm absorption in Fig. 3B suggests that a significant fraction of lipid assembled into vesicles devoid of RCs. Figure 4 illustrates the results of this indirect way of assessing the distribution

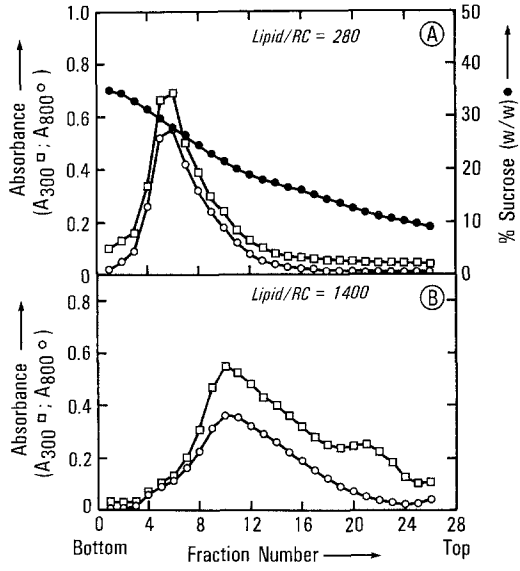

Fig. 3. Sucrose density gradient centrifugation of RC vesicles reconstituted at different lipid-to-protein ratios. RC vesicles were reconstituted by sonication from asolectin and LDAO-depleted RCs at a molar ratio of (A) 280 and (B) 1400. Absorbance measured at 300 (□) and 800 (○) nm.

of vesicles over density gradients (the absorption at 300 nm), and direct measurements of radio-labeled lipid. At low lipid-to-RC ratios, there is good correspondence between the two assays while, at higher ratios, the absorbance measurements underestimate the amount of lipid in the top of the gradient.

The measurements of the density of reconstituted RC vesicles are summarized in Fig. 5. In most of the gradients a relatively narrow band of the reconstituted vesicles was obtained, in which the maximal absorbance at 300 and 800 nm usually coincided; therefore, the density of the fraction with the maximal absorption at 800 nm (which in most cases matches the average density of the total sample) was plotted (open symbols). In addition, gradients were run to which either LDAO-depleted, LDAO-containing RCs, or sonicated asolectin vesicles were applied. The broken line in Fig. 5 shows the density of the lipid vesicles, whereas the values at zero lipid/RC molar ratio represent the density of the two RC preparations. The latter values are mutually consistent assuming that the density of the bound LDAO is 1.0 g/cm^3. Next, the predicted value for the average density of the separate samples of reconstituted vesicles was calculated using the measured values of the density of the individual components (i.e., the two RC preparations and asolectin vesicles) and their added amounts in the reconstitution (closed symbols). With vesicles reconstituted from LDAO-depleted RCs (circles)

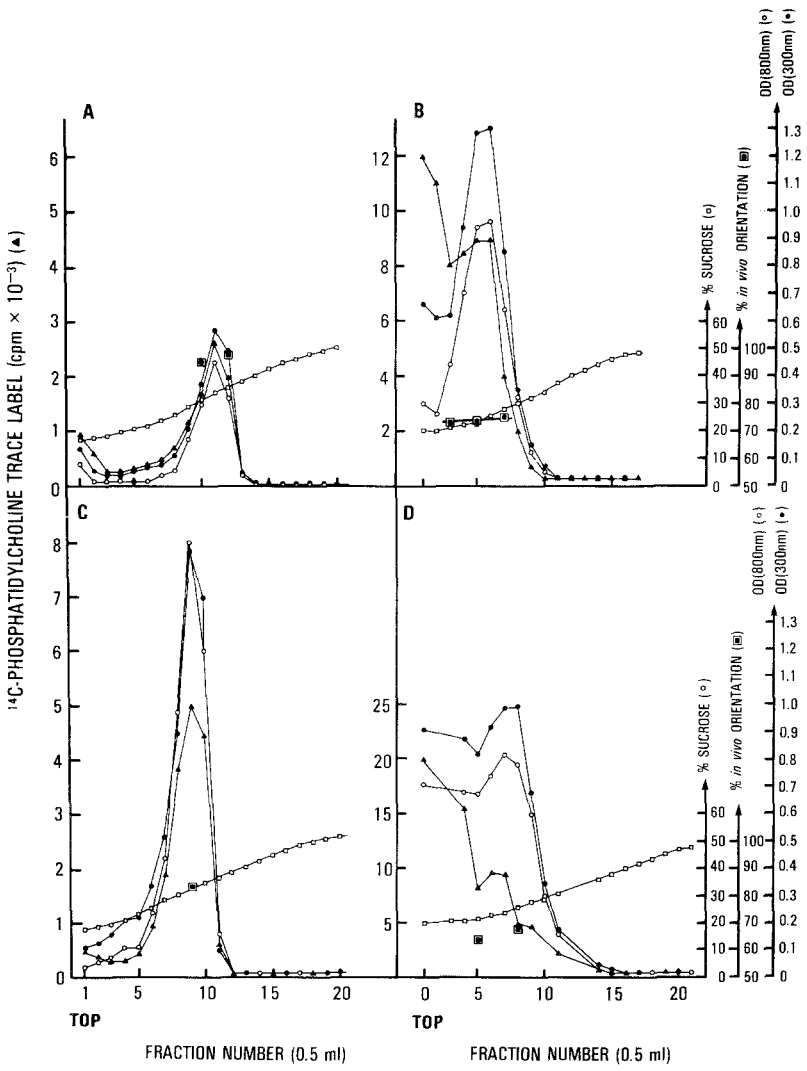

Fig. 4. Direct and indirect measurements of the distribution of lipid in sucrose gradients. (A, B) Vesicles were reconstituted by sonication from asolectin and LDAO-containing RCs, according to the standard procedure (see Methods), except that 6.25 nCi of labeled lecithin per mg asolectin was added to asolectin in hexane, and this mixture was suspended in 10 mM Tris pH 8.0, instead of the 200 mM KCl. The molar lipid-to-protein ratio was 140 in (A) and 560 in (B). (C, D) Vesicles were reconstituted by sonication from asolectin, with the same amount of radiolabeled lipid added as in (A) and (B) and LDAO-depleted RCs according to the standard procedure (see Methods) with molar lipid ratios of 140 and 560. Sucrose gradients from 10 to 70% sucrose were used for all samples. Open squares: % sucrose; closed squares: RC orientation; open circles: absorbance at 800 nm; closed circles: absorbance at 300 nm; triangles: amount of radioactivity per 0.5 ml fraction of the sucrose gradient. The percentage *in vivo* orientation in the four samples before fractionation was 97, 73, 82, and 68% for sample A, B, C, and D respectively.

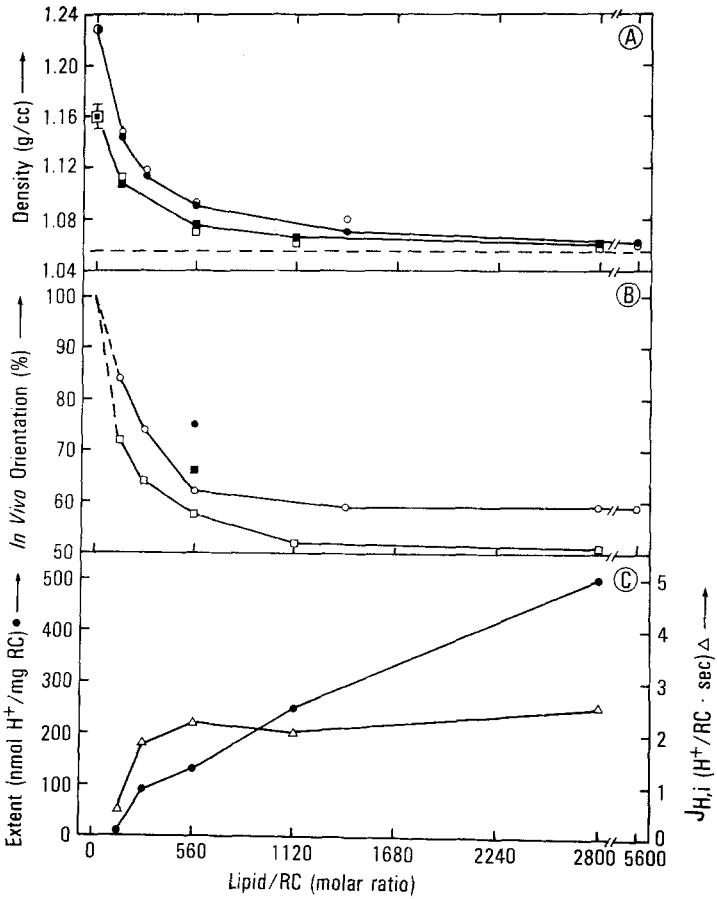

Fig. 5. Characterization of reconstituted RC vesicles. (A) Average density of sonicated RC vesicles as a function of the lipid/RC ratio during reconstitution. Squares: vesicles reconstituted at various lipid/RC ratios from asolection and LDAO-containing RCs; circles: vesicles from asolectin and LDAO-depleted RCs. Open symbols: measured densities; closed symbols: calculated densities. The broken line indicates the density at which sonicated asolectin vesicles band in the sucrose gradient. An average molecular weight of 700 and 95,000 for lipid and RCs was used, respectively. (B) Orientation of RCs in reconstituted vesicles as a function of the lipid/RC ratio during reconstitution. The filled symbols represent an experiment in which 25% (w/w) cholesterol was added to asolectin before the lipids were suspended in 200 mM KCl. (C) Initial rate and extent of light-dependent proton translocation in reconstituted RC vesicles as a function of the lipid/RC ratio during reconstitution. Circles: extent of proton extrusion; triangles: initial rate of proton extrusion. Sonicated vesicles were used with LDAO-containing RCs.

and with those from LDAO-containing RCs (squares), there is good agreement between these two values. This indicates that during the 10-min sonication in the reconstitution procedure, random lateral association between lipid and protein occurred. The bar indicated for the density of the LDAO-containing RCs reflects the observation that, in this case, two major bands in the sucrose gradient were visually identified but were not resolved upon fractionation. With LDAO-depleted RCs, one sharp band was obtained at a density 1.23 g/cm^3. With RC vesicles reconstituted at a molar lipid-to-protein ratio lower than 560, a fraction of RC molecules was not incorporated into vesicles: at a ratio of 280, this was $\leqslant 10\%$, and at a ratio of 140, it increased to 20 and 50% for LDAO-containing and LDAO-depleted RCs, respectively. These values were estimated from absorbance measurements on a "pellet" of RC-containing material that remained at the bottom of the tube after fractionation and that was recovered in a small volume of LDAO-containing buffer. This material most probably represents free RCs since all the cytochrome *c* binding sites of the RCs were available for cytochrome *c* in the external medium. Figure 5C illustrates the results of proton translocation measurements with vesicles reconstituted from asolectin and LDAO-containing RCs (for the description of the procedure for measurement of proton translocation, see Hellingwerf, 1987). Except for the lowest lipid/RC ratio, where the relatively high content of LDAO impairs the barrier properties of the membrane (data not shown), there is a linear increase in the extent of proton transport with lipid/RC ratio, whereas the initial rate of proton translocation remained constant. Similar experiments with vesicles, reconstituted from LDAO-depleted RCs, showed an approximately linear increase in the extent of proton translocation with lipid-to-protein ratio. The curve was slightly sigmoidal at the lower lipid/RC ratios, presumably on account of nonvesicle-incorporated RCs (compare Fig. 5A, C).

The Size of Reconstituted RC Vesicles

The size distribution of the various vesicle preparations was estimated from light-scattering measurements. The weight-average size of RC vesicles prepared from LDAO-depleted RCs decreases from 200 to 100 nm when the molar lipid-to-RC ratio varies from 140 to 5600. After centrifugation at 60,000 $\times$ g for 4 min in 50-μl samples (a procedure during which particles larger than 10^6 Dalton were pelleted; Z. Kam, personal communication), vesicles with an approximate diameter of 100 nm are present in all samples. The percentage of RCs removed during this centrifugation gradually decreases from 30% at the lowest lipid/RC ratio to an insignificant amount at a ratio of 5600. For vesicles prepared from LDAO-containing RCs, the average size of the vesicles decreased from 200 to 80 nm. In these

preparations, even less RCs were pelleted upon centrifugation and vesicles with an average diameter of 80 nm were present in all samples after centrifugation.

Figure 6 shows freeze-fracture electron micrographs of vesicles reconstituted from LDAO-depleted RCs in a molar ratio of lipid to protein of 560. In agreement with the light-scatter measurements, both fairly large ($\geqslant$ 200 nm diameter) and a large number of vesicles of more or less uniform size (approximately 100 nm diameter) are distinguished. The density of intramembranous particles in the fracture plane of the membrane appears to be the same in the large and small vesicles. In addition, only a limited number of vesicles are devoid of intramembranous particles. The diameter of these particles is 8–9 nm on the average; occasionally, particles of 13 nm are seen. The size heterogeneity of the reconstituted RC vesicles is also observable in their elution pattern when subjected to molecular sieve chromatography on BioGel A50 (Fig. 7). In addition, all subfractions within one sample of reconstituted vesicles have approximately a constant lipid-to-protein ratio (as determined from the ratio of radioactivity over absorbance at 800 nm), in spite of the size heterogeneity within the sample. Absolute values for the size of the reconstituted vesicles are difficult to derive from this type of experiment, since the largest vesicles elute in the void volume. However, the light scattering data of individual fractions from such columns are in agreement with the data described above.

Parameters That Affect the Orientation of Reconstituted RCs

For functional studies on reconstituted RCs, it is crucial to know the orientation of the RCs with respect to the plane of the membrane. Therefore, this parameter was measured. As shown in Fig. 2, there was no measurable change in RC orientation as a function of sonication time. The density of the subfractions of vesicles within one sample did slightly affect the net orientation of the RCs: a slight increase of net orientation with density is observed (Fig. 4). Furthermore, there was a good correlation between the measured RC orientation in the individual fractions and the overall orientation in the sample. Figure 5B shows the result of measurement of the orientation of RCs in vesicles reconstituted at different lipid-to-protein ratios. In general (see Figs. 2 and 4) an excess of RCs had the cytochrome *c* binding site on the exterior of the vesicles (this is here denoted as *in vivo* orientation, since in the intact bacterium, this side of the RCs is at the exterior of the cytoplasmic membrane). Reconstituted vesicles with or without prior LDAO depletion of the RCs showed differences, particularly at high lipid/RC ratios: When the reconstitution was performed with RCs that have their full complement of LDAO, no net orientation of RCs at the highest lipid/RC ratios was detected

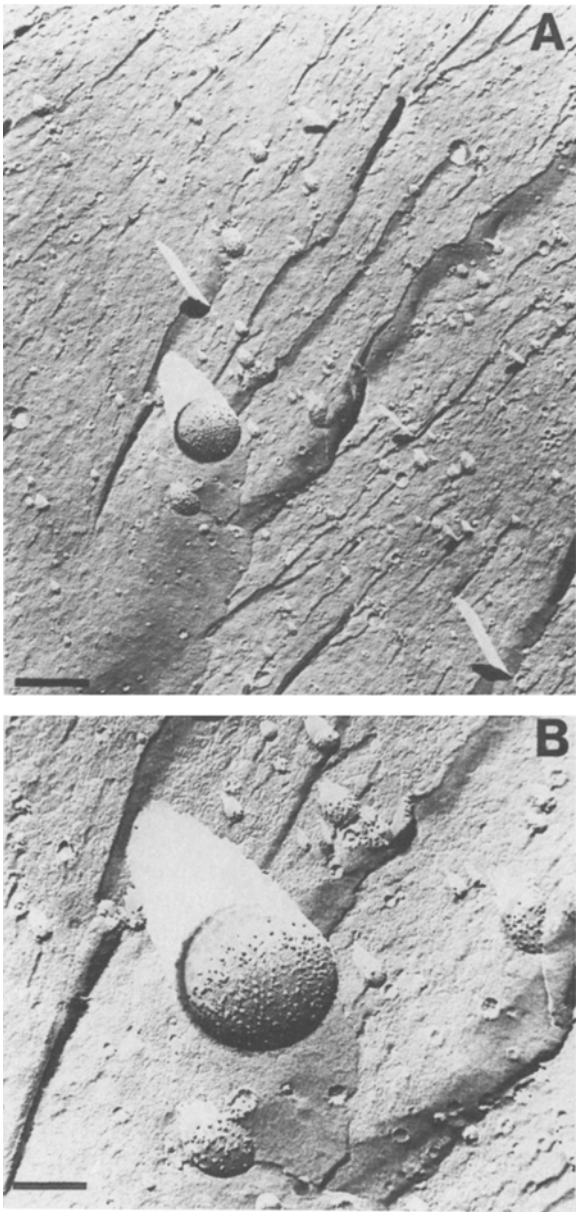

Fig. 6. Electron micrographs of freeze fracture replicas of reconstituted RC vesicles. Vesicles were reconstituted from asolectin and LDAO-depleted RCs (molar ratio 560) by sonication. The bars represent 800 and 160 nm, respectively.

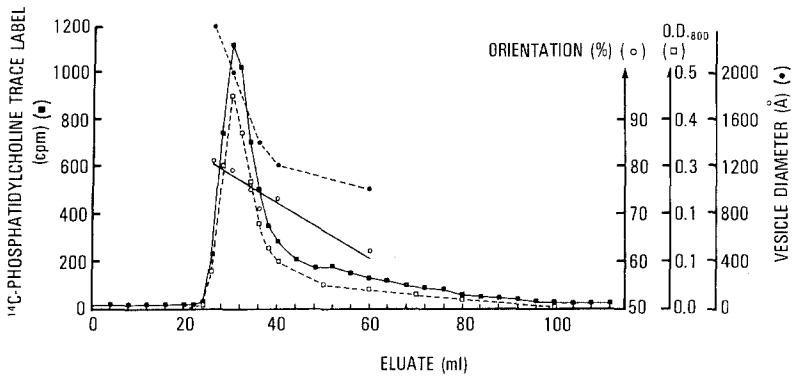

Fig. 7. Molecular sieve chromatography of reconstituted RC vesicles. Vesicles were reconstituted by sonication from asolectin (to which 0.6 nCi of the labeled lecithin per mg asolectin was added in hexane) and LDAO-depleted RCs (molar ratio 560) according to the standard procedure (see Methods). A 0.4-ml portion of this suspension was applied to a Biogel A 50 column (total volume 200 ml; effective volume 150 ml), which was run at room temperature (21°C). Fractions of 2 ml were collected and assayed as described under Methods. From the amount of radioactivity before and after vesicle fractionation, a recovery of > 95% of the lipid was estimated.

(i.e., 50% *in vivo* orientation) whereas the corresponding vesicles from LDAO-depleted RCs show a net orientation of 20% (thus 60% *in vivo* orientation). At low ratios (i.e., 140 and 280), the orientation is obscured by RCs that are not incorporated into vesicles (Fig. 5; see above). However, it can be inferred that on lowering the lipid/RC ratio, the net orientation of the RCs increases to ~40% (= 70% *in vivo* orientation). Nevertheless, it is clear that, under these conditions, a complete unidirectional orientation of the RCs is not attained.

The size of the reconstituted vesicles has an effect on the orientation of the RCs (Fig. 7) which decreases from 82% in the largest vesicles to 64% in the smallest vesicle population reconstituted at a lipid-to-protein ratio of 560. Cholesterol, when mixed with the lipids before reconstitution, slightly enhances the unidirectional orientation of the RCs (Table I), both when the sonication or the cholatedialysis procedure for reconstitution was used. These two procedures (lines A and F or D and H) do not yield large differences in the orientation of RCs. The low values of the extent of proton translocation in lines E and G (at a low lipid/RC ratio) indicate that a significant fraction of the RCs are not incorporated into vesicles, and that the (relatively high) orientation is an overestimation. Table II shows that lowering the salt concentration during reconstitution produces a significant increase in orientation of the reconstituted RCs such that, at very low ionic strength, an almost unidirectional orientation of the RCs was obtained. The experiments summarized in Fig. 4 show that in this case, even at the lowest lipid/RC ratio

Table I. Orientation of Reconstituted RCs and Extent of Proton Translocation in Vesicles, Reconstituted by Different Reconstitution Procedures with and without Cholesterol[a]

Exp.	Ratio	Proc.	Chol.	LDAO	% Or.	Ext.
A	560	s	−	+	57	130
B	560	s	−	−	62	92
C	560	s	+	+	66	110
D	560	s	+	−	75	67
E	140	d	−		91	< 3
F	560	d	−		59	70
G	140	d	+		84	10
H	560	d	+		89	24

[a]Ratio: molar ratio of lipid in asolectin and RCs in the reconstitution. Proc.: reconstitution procedure used; s: sonication; d: dialysis. Chol.: where indicated, cholesterol was mixed with asolectin (1/4, w/w) in hexane, prior to the reconstitution. LDAO: + and − indicate the use of LDAO-containing and LDAO-depleted RCs in the reconstitution. % Or.: percentage of the RCs with the cytochrome *c* binding site at the exterior of the vesicles. Ext.: extent of light-dependent proton translocation (in nmol H^+/mg RCs).

Table II. The Effect of Salt Concentration during Reconstitution on the Orientation of the RCs[a]

KCl concentration	Percent orientation
0	93
10	90
100	70
500	55

[a]Vesicles were reconstituted from asolectin and LDAO-containing RCs, as described under Methods, except that asolectin was suspended in 10 mM Tris, pH 8.0, 0.1 ml of both RCs and the asolectin suspension were mixed with 0.2 ml of a concentrated KCl solution to yield the indicated KCl concentration.

(140), all RCs were incorporated in closed lipid vesicles, as shown by the comigration of the two components in the sucrose-density gradient.

Discussion

The results summarized in this paper demonstrate that a 10-min sonication leads to a virtually complete incorporation of RCs into lipid bilayers, if enough lipid is provided. At low ionic strength this is valid down to a lipid/RC ratio of 140; at higher ionic strength the lipid requirement is somewhat larger (see Figs. 3–5). Under optimal conditions (with a lipid/RC ratio between 500 and 1000) uniform vesicles are obtained with respect to density. If, in addition, the reconstitution is performed at low ionic strength, the RCs will be highly orientated (Table II).

The mechanism that underlies the process of incorporation of RCs in the reconstituted membranes is still unknown. Therefore, a discussion of the factors that determine the orientation of the reconstituted RC molecules remains necessarily speculative. The available evidence is in agreement with a suggestion for the reconstitution of bacteriorhodopsin (Hellingwerf, 1979). Accordingly, the orientation of the reconstituted protein is determined primarily by the hydrophobicity/hydrophilicity characteristics of the two opposing surfaces of the protein. In RCs, the most hydrophobic surface appears to be the one that possesses the quinone binding site (Blatt *et al.*, 1983; Pachence *et al.*, 1983). This region binds initially to the relatively hydrophobic surface of pre-existing vesicles, and ends up at the interior surface of the membrane. This effect is particularly prominent at low ionic strength, when electrostatic repulsion and/or attraction is most effective (compare Table II). The asymmetric mass distribution of the RCs, however, may promote randomization as the curvature of the vesicles increases (i.e., as their size decreases). The results summarized in Fig. 7 and Table II support these suggestions. The observed correlation between decreasing vesicle size and increased RC scrambling is in agreement with other reports on the orientation of RC in reconstituted proteoliposomes (Sadler *et al.*, 1984; Iba *et al.*, 1984). Sadler *et al.* observed approximately 95% *in vivo* orientation of RCs reconstituted with egg lecithin with a reconstitution procedure that combines sonication and dialysis. The resulting liposomes had an average diameter of almost 200 nm and were prepared at a relatively high protein-to-lipid ratio. Their results largely agree with the results of Pachence *et al.* (1979, 1981, 1983). Iba *et al.* reconstituted RC liposomes with asolectin at a high lipid-to-protein ratio. Upon fractionation of this mixture with sedimentation velocity centrifugation, they also observed a correlation between vesicle size and RC orientation. Overfield and Wraight (1980a, 1980b) observed a dependence of RC orientation on net charge of the lipids used in the reconstitution. A high degree of net orientation (90% *in vivo* orientation) was obtained upon reconstitution with phosphatidylserine, whereas the orientation was random in neutral lecithins. Asolectin contains a significant fraction of negatively charged lipids (Kagawa and Racker, 1971).

Dutton and co-workers (Pachence *et al.*, 1979, 1981, 1983) presented an extensive investigation on the structural properties of reconstituted RC vesicles. They used a different reconstitution procedure with egg lecithin as the lipid (Pachence *et al.*, 1979). They observed that RC orientation was independent of the lipid/RC ratio (Pachence *et al.*, 1979). Their conclusion depends on the ability to discriminate between the occurrence of closed vesicles and lipoprotein complexes. Our results indicate that with asolectin low lipid/RC ratios would promote the formation of lipoprotein complexes rather than closed vesicles. Lipoprotein complexes exhibit an apparent

100% *in vivo* orientation, and, therefore, complicate the orientation assay. Such ambiguity was dealt with in our study through the use of equilibrium centrifugation on sucrose gradients. An alternative procedure includes cytochrome *c* in the RC vesicles during reconstitution. However, contradictory results have been reported (see Casey *et al.*, 1982) and investigations on the orientation of cytochrome *c* oxidase showed that the mere presence of cytochrome *c* during reconstitution led to randomization of the reconstituted enzyme (Carroll and Racker, 1977). Rich and Heathcote (1984) have recently reported a method to assay the presence of the quinone-binding site of the RCs behind the diffusion barrier of a closed membrane. This method may prove useful to ascertain the presence or absence of lipoprotein complexes.

A survey of the literature on the properties of reconstituted proteoliposomes leads us to conclude that there are no general rules for their structure. Even with one protein and one lipid species the results vary widely (e.g., Hellingwerf *et al.*, 1978; Hellingwerf, 1979). To illustrate this point, two recent studies are worth mentioning: Casey *et al.* (1982) studies the orientation of cytochrome *c* oxidase reconstituted with asolectin. They report that (i) reconstitution via sonication yields lower protein orientation than via cholate dialysis; (ii) addition of cholesterol decreased orientation; (iii) orientation was independent of vesicle size. These results are opposite to those reported here. Anholt *et al.* (1982) describe a decrease in orientation of the *Torpedo* acetylcholine receptor upon addition of cholesterol concomitant with a slight increase in vesicle size. Furthermore, bacteriorhodopsin and the acetylcholine receptor associate nonrandomly with lipid during reconstitution (Anholt *et al.*, 1982; Hellingwerf, 1979). The parameter(s) that are responsible for these differences are currently unresolved. Much further work is required before the mechanism of the reconstitution of intrinsic membrane proteins can be interpreted at a molecular level. It can be anticipated that the information on the three-dimensional structure of RCs from purple bacteria (e.g., Deisenhofer *et al.*, 1985) that has recently become available will be very important in further attempts to resolve this mechanism.

Conclusions

During reconstitution of RCs and asolectin via sonication, lipid and protein associate at random, so that, at an optimal lipid/RC ratio (500 to 1000), vesicles of uniform density are formed. The orientation of the reconstituted RCs was observed to be nonrandom. The reconstituted vesicles are heterogeneous in size. The largest vesicles exhibit the highest net RC orientation (with the cytochrome *c* binding site facing the vesicle exterior). In addition, this characterization provides guidelines for the optimal choice of

reconstitution conditions in further studies of the mechanisms of light-dependent electron transfer and protonmotive force generation in RCs.

Acknowledgments

This research was performed at the University of California San Diego in collaboration with George Feher and Mauricio Montal. I thank E. Abresch for the isolation of RCs, Z. Kam for performing the light-scattering measurements, and M. Ellisman for the electron microscopic study.

References

Anholt, R., Fredkin, D. R., Deerinck, T., Ellisman, M. H., Montal, M., and Lindstrom, J. (1982). *J. Biol. Chem.* **257**, 7122–7134.

Blatt, Y., Gopher, A., Montal, M., and Feher, G. (1983). *Biophys. J.* **41**, 121a.

Carroll, R. C., and Racker, E. (1977). *J. Biol. Chem.* **252**, 6981–6990.

Casey, R. P., Ariano, B. H., and Azzi, A. (1982). *Eur. J. Biochem.* **122**, 313–318.

Crofts, A. R., Crowther, D., Celis, H., Almanza de Celis, S., and Tierney, G. (1977). *Biochem Soc. Trans.* **5**, 491–495.

Darszon, A., Vandenberg, C. A., Schönfeld, M., Ellisman, M. H., Spitzer, N. C., and Montal, M. (1980). *Proc. Natl. Acad. Sci. USA* **77**, 239–243.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985). *Nature* (*London*) **318**, 618–624.

Dutton, P. L., Mueller, P., O'Keefe, D. P., Packham, N. K., Prince, R. C., and Tiede, D. M. (1982). *Curr. Top. Membr. Transport* **16**, 323–343.

Feher, G., and Okamura, M. Y. (1978) In *The Photosynthetic Bacteria* (Clayton, R. K., and Sistrom, W. R., eds.), Plenum Press, New York, Chapter 19, pp. 349–386.

Hellingwerf, K. J. (1979). Structural and Functional Studies on Lipid Vesicles Containing Bacteriorhodopsin, Ph.D. Thesis, Univ. of Amsterdam, Veenstra-Visser, Groningen.

Hellingwerf, K. J. (1984). In *Advances in Photosynthesis Research* (Sybesma, C., ed.), Martinus Nijhoff/Dr. W. Junk, Publishers, The Hague, Vol. II, pp. 367–370.

Hellingwerf, K. J. (1987). *J. Bioenerg. Biomembr.* **19**, 225–238.

Hellingwerf, K. J., and Konings, W. N. (1985). *Adv. Microbial Physiol.* **26**, 125–154.

Hellingwerf, K. J., Scholte, B. J., and Van Dam, K. (1978). *Biochim. Biophys. Acta* **513**, 66–77.

Holloway, P. W. (1973). *Anal. Biochem.* **53**, 304–308.

Iba, K., Takamiya, K.-I., Aruta, H., Toh, Y., and Nishimura, M. (1984). *J. Biochem.* **96**, 1823–1830.

Kagawa, Y., and Racker, E. (1971). *J. Biol. Chem.* **246**, 5477–5487.

Konings, W. N., Hellingwerf, K. J., and Robillard, G. T. (1982). In *Comprehensive Biochemistry* (Bonting, S. L., and de Pont, J. J. H. H. M., eds.), Elsevier/North Holland, Amsterdam, Chapter 10, pp. 257–283.

Likhtenstein, G. I., Kulikov, A. V., Kotelnikov, A. I., and Bogatyrenko, V. R. (1982). *Photobiochem. Photobiophys.* **3**, 337–344.

McElroy, J. D., Mauzerall, D. C. and Feher, G. (1974). *Biochim. Biophys. Acta* **333**, 261–277.

Mitchell, P. (1968). *Chemiosmotic Coupling and Energy Transduction*, Glynn Research, Bodmin, Cornwall, England.

Okamura, M. Y., Feher, G., and Nelson, N. (1982). In *Energy Conversion by Plants and Bacteria* (Govindjee, ed.), Academic Press, New York, Vol. I, pp. 195–274.

Overfield, R. E., and Wraight, C. A. (1980a). *Biochemistry* **19**, 3322–3327.

Overfield, R. E., and Wraight, C. A. (1980b). *Biochemistry* **19**, 3328–3334.

Pachence, J. M., Dutton, P. L., and Blasie, J. K. (1979). *Biochim. Biophys. Acta* **548**, 348–373.

Pachence, J. M., Dutton, P. L., and Blasie, J. K. (1981). *Biochim. Biophys. Acta* **635**, 267–283.
Pachence, J. M., Dutton, P. L., and Blasie, J. K. (1983). *Biochim. Biophys. Acta* **724**, 6–19.
Racker, E. (1973). *Biochem. Biophys. Res. Commun.* **55**, 224–230.
Rich, P. R., and Heathcote, P. (1984). *Biochim. Biophys. Acta* **725**, 332–340.
Sadler, D. M., Rivas, E., Gulik-Krzywicki, T., and Reiss-Husson, F. (1984). *Biochemistry* **23**, 2704–2712.
Schönfeld, M., Montal, M., and Feher, G. (1979). *Proc. Natl. Acad. Sci. USA* **76**, 6351–6355.
Schönfeld, M., Montal, M., and Feher, G. (1980). *Biochemistry* **19**, 1535–1542.
Valkirs, G. E., and Feher, G. (1982). *J. Cell Biol.* **95**, 179–188.