Physicochemical Properties of Detergent-Solubilized Photochemical Reaction Centers from Two Strains of *Rhodopseudomonas spheroides*†

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ABSTRACT: Reaction centers purified from *Rhodopseudomonas spheroides* (wild-type and R 26 strains) were characterized in detergent solutions by sedimentation velocity and sedimentation equilibrium analysis in detergent buffers containing various amounts of $H_2{}^{18}O$. In the range of detergent concentrations studied (0.5–2.0 mg of dodecyldimethylamine *N*-oxide per mL), the detergent–reaction center complexes are monodisperse; sedimentation equilibrium experiments in H_2O–$H_2{}^{18}O$ buffers revealed particle weights of 138 900 and 140 700 for the R 26 and wild-type reaction center complexes, respectively. Under these conditions 0.67 and 0.58 g of dodecyldimethylamine *N*-oxide were bound per g of protein in R 26 and wild-type preparations, respectively, as determined by chromatography using ^{14}C-labeled detergent. From these data one may deduce that the reaction centers are present as monomers and that the molecular weight of their protein moiety is 80 000. Another independent measure of the protein molecular weight was performed by adjusting the solvent density so as to blank out the contribution of detergent to the sedimentation potential; a value of 87 000 was thus obtained. Hydrodynamic properties of the detergent–reaction center complexes were derived from sedimentation velocity and sedimentation equilibrium data. The $s_{20,w}$ values are 3.9 and 3.6 S and the Stokes radii are 41 and 47 Å for R 26 and wild-type complexes, respectively. In both detergent–protein complexes, the ratio of the Stokes radius to the minimum possible radius falls within the range of values compatible with that of globular-shaped particles.

Recently much progress has been made in the characterization of photochemical reaction centers isolated from several bacterial species, especially from *Rhodopseudomonas spheroides* (*R. spheroides*).[1] These complexes, constituted by a multichain integral membrane protein and a number of associated molecules including bacteriochlorophyll (BChl), bacteriopheophytin (Bpheo), ubiquinone (UQ), iron, and possibly carotenoid, are the simplest units presently known which are capable of performing in vitro the primary photochemical reactions. Although detailed information exists concerning the nature and the temporal sequence of these processes, much less is known about the molecular architecture of the complex and about the structure of the protein. Indeed, the protein molecular weight has not yet been accurately determined (Feher & Okamura, 1978; Gingras, 1978).

When isolated from *R. spheroides* R 26 membranes by mild treatment with lauryldimethylamine *N*-oxide (LDAO), the reaction center protein is composed of three polypeptide chains called L, M, and H, probably present in a 1:1:1 molar ratio (Clayton & Haselkorn, 1972; Okamura et al., 1974; Steiner et al., 1974). The electrophoretic mobilities of these subunits in sodium dodecyl sulfate–polyacrylamide gels correspond to those of polypeptides having molecular weights of 21 000, 24 000, and 28 000, respectively. From these data one could estimate the minimal molecular weight of the protein to be 73 000 (Okamura et al., 1974). It is, however, well documented that estimation of molecular weights from $NaDodSO_4$ gels can lead to serious error in the case of membrane proteins or glycoproteins (Rizzolo et al., 1976; Tanford & Reynolds, 1976). Another value for the molecular weight, 90 000, was obtained from the dry weight (Feher, 1971) and the molecular extinction coefficient (Reed & Peters, 1972; Straley et al., 1973). More recently, the minimum molecular weight of the L subunit was revised to a value of 28 000, on the basis of the amino acid composition and the integral residue method (Feher & Okamura, 1978). In reaction centers isolated with LDAO from wild-type *R. spheroides*, three polypeptide chains with the same electrophoretic mobilities as those of the mutant are found (Jolchine & Reiss-Husson, 1974).

In the present work, we have accurately measured the molecular weight of the protein of reaction centers isolated from the two strains of *R. spheroides* (wild-type Y and R 26 mutant), by sedimentation methods. This determination, as those relevant to other hydrophobic proteins in detergent solution, requires either that the amount of bound detergent be known (Tanford et al., 1974) or that the contribution of detergent to the sedimentation potential be blanked out by adjusting the solvent density to a value equal to the inverse of the detergent specific volume (le Maire et al., 1976a; Reynolds & Tanford, 1976). Both approaches have been used in the present study. The precise amount of detergent bound to the complex has been determined by chromatographic procedures; the mass of the reaction center–detergent complex and its partial specific volume have been calculated from sedimentation equilibrium in solvents of different densities, as well as the molecular weight of the protein moiety. We have also shown that the shape of the complexes is globular and defined the conditions to obtain concentrated monodispersed solutions suitable for more detailed structural analysis of these complexes.

Experimental Procedures

Chemicals. Commercial LDAO was a gift from Onyx Chemicals and was used without further purification. Chemically pure dodecyldimethylamine oxide (DDAO) synthetized from redistilled dodecylamine as described by Applebury et al. (1974) was obtained from the CEA (Saclay, France) in its cold and labeled ([^{14}C]DDAO; 24 mCi/mmol)

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[1] Abbreviations used: *R. spheroides*, *Rhodopseudomonas spheroides*; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; LDAO, commercial dodecyldimethylamine *N*-oxide; DDAO, dodecyldimethylamine *N*-oxide; BChl, bacteriochlorophyll; Bpheo, bacteriopheophytin; UQ, ubiquinone.

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forms; the radioactive compound cochromatographed with the unlabeled one in thin-layer plates by using methanol–chloroform–ammonia (Pelka & Metcalfe, 1965) and acetonitrile–acetic acid, 98:2 (v/v), as developing solvents, as well as in gel column chromatography. Catalase (30 μg/L) was systematically added to LDAO or DDAO solutions, to remove any traces of H_2O_2 present in the detergent (Straley et al., 1973). Commercially available DDAO (Fluka) was found not to be reagent grade and also to have a strong oxidizing power; its use is not recommendable.

Preparation of Reaction Centers. Wild-type (strain Y) *R. spheroides* cells were grown anaerobically in the light in the "L, 17 μM Fe" medium as already described (Reiss-Husson et al., 1971). R 26 cells were grown similarly, except that a modified Hunter medium (Cohen-Bazire et al., 1957) was used. R 26 reaction centers were isolated with LDAO and purified according to Okamura et al. (1974), except that a linear NaCl gradient was used in the DEAE-cellulose chromatography instead of a stepwise elution. Wild-type reaction centers were isolated with LDAO and purified as already described (Jolchine & Reiss-Husson, 1974), with an additional purification by DEAE-cellulose chromatography (as above) after the agarose 6 B chromatography. Both reaction center preparations were finally concentrated by ultrafiltration under N_2 on a Amicon PM 30 membrane (up to ~4 mg protein per mL) in a 10 mM Tris-HCl–0.1 mM EDTA buffer, pH 8.0, in presence of 1 mg of LDAO per mL.

In these experiments we have chosen as a criterion of purity of the reaction center samples the ratio of absorbances A_{802nm}/A_{280nm}, as emphasized by Okamura et al. (1974). In the protein peak eluted by chromatography, the minimum value of this ratio was 1.22 ± 0.01 for the R 26 reaction centers and 1.16 ± 0.02 for the wild-type one. Only fractions presenting this ratio were used for the determination of the detergent–protein binding; samples of the same purity were employed for the analytical centrifugation experiments and the phosphorus analysis.

Determination of the Amount of Bound DDAO. This determination was performed with a chemically homogeneous detergent, i.e., DDAO. LDAO (whose fatty acid chain composition is heterogeneous) was thus exchanged against DDAO by gel filtration, ion-exchange chromatography, or a combination of these techniques.

(*a*) *Gel Filtration [According to Hummel & Dreyer (1962)].* A Sepharose 6B-CL column (diameter 1.5 cm; height 90 cm) was equilibrated with buffer (10 mM Tes–50 mM NaCl, pH 7.5) containing a suitable concentration C_0 of labeled DDAO. A reaction center sample prepared in LDAO (see above) was applied and eluted at a flow rate of 5 mL/h with the labeled DDAO containing buffer. This resulted in the exchange of LDAO by DDAO in the reaction center complex.

The absorbance of the eluate was followed at 802 and 280 nm, on a Cary 14 spectrophotometer.

Fractions of ~1.2 mL were collected; 100-μL aliquots were taken in duplicate from all fractions for scintillation counting and for protein determination. In any fraction containing C_p grams per milliliter of reaction center protein and given n cpm, the binding of [^{14}C]DDAO to reaction center protein was calculated as

$$\frac{C_0}{C_p}\left(\frac{n - \text{average cpm of base line}}{\text{average cpm of base line}}\right) \quad (1)$$

(*b*) *Ion-Exchange Chromatography.* Hydroxylapatite (HT, Bio Rad) was packed as a column just big enough to adsorb the reaction center sample (for a 10-mg protein samples, we used about 3 cm^3 of hydroxylapatite) and equilibrated with the buffer containing [^{14}C]DDAO. A reaction center sample prepared in LDAO was adsorbed, and LDAO was exchanged for DDAO by washing with the [^{14}C]DDAO buffer; the eluate was monitored for a stable base line of radioactivity. The reaction center was then eluted sharply with 0.15 M potassium phosphate in the [^{14}C]DDAO buffer. Fractions (0.2 mL) were collected, from which 50-μL aliquots were taken for scintillation counting. Aliquots (20 μL) from the eluted reaction center fractions were used for protein determination. All the aliquots were taken in duplicate. Binding of DDAO was determined by eq 1; phosphate present in the reaction center fractions was eventually eliminated by dialysis against the radiolabeled buffer and the binding ratio was recalculated afterward.

(*c*) *Combined Ion-Exchange–Gel Filtration Chromatography.* Sephadex G-25M was packed in a short column (1.6-cm diameter; 3.0-cm height) and covered with a disk of filter paper; hydroxylapatite (1 cm^3) was then gently layered onto it. The double column was equilibrated with [^{14}C]DDAO buffer, and the experiment was carried out as described above (cf. Ion-Exchange Chromatography) except that only a "pulse" (~300 μL) of 0.15 M potassium phosphate in [^{14}C]DDAO buffer was used to elute the reaction center, immediately followed by the equilibrium buffer. This allows the elution of a sharp and concentrated peak of reaction center complex free of inorganic phosphate.

Sedimentation Equilibrium in Solvents of Various Densities. We have used this method, originally suggested by Edelstein & Schachman (1967) in studies of water-soluble proteins, for obtaining the particle weight M^* and the specific volume $\bar{v}^*$ of the entire complex formed by protein, pigments, and bound detergents. The variation of the density of the solvent was obtained by using different amounts of $H_2{}^{18}O$ in the DDAO buffer (10 mM Tes–50 mM NaCl–DDAO, 1 g/L, pH 7,5). A Model E analytical ultracentrifuge, equipped with a photoelectric scanner, was used; samples were placed in Yphantis cells in a An-G rotor. The absorption of the samples at 280 or 365 nm was measured against that of the solvent at 20 °C after equilibration at speeds varying from 12 000 to 20 000 rpm. In order to ascertain whether regression lines obtained from equilibrium plots were representative of all reaction center molecules in solution, we calculated recovery as previously described (le Maire et al., 1978).

The slope of equilibrium plots ln c vs. r^2 (c is concentration and r is radial distance) is a quantity which is formally equal to $M^*(1 - \bar{v}^*\rho)$, ρ being the density of the solvent; thus, by plotting this quantity versus ρ, a linear variation should be obtained, yielding the value of $\bar{v}^*$ by extrapolation to $M^*(1 - \bar{v}^*\rho) = 0$; the particle weight of the complex, M^*, is calculated from the slope of this plot. This particle weight is related to the protein molecular weight, M_p, by eq (2) (Tanford et al., 1974):

$$M^* = M_p(1 + \delta_D + \sum_i \delta_i) \quad (2)$$

where δ_D is the amount of bound DDAO expressed as grams of bound material per gram of protein, and δ_i are amounts of other bound components i (pigments, ubiquinone, etc.) similarly expressed.

Furthermore, at any value of the solvent density ρ, the general relation (eq 3) may be used:

$$M^*(1 - \bar{v}^*\rho) = M_p[(1 - \bar{v}_p\rho) + \delta_D(1 - \bar{v}_D\rho) + \sum_i \delta_i(1 - \bar{v}_i\rho)] \quad (3)$$

Table I: Chemical Components of Reaction Centers besides Polypeptide Chains

component	M_i	n_i (mol/mol of reaction center) Y strain	R 26 strain	$\bar{v}$ (cm^3/g)
bacteriochlorophyll	911.8	4[c]	4[a]	0.799[e]
bacteriopheophytin	889.5	2[c]	2[a]	0.800[f]
ubiquinone Q-10	863.4	2[b]	2[b]	1.023[e]
Fe	55.8	1[a]	1[a]	0.13[e]
spheroidene	568.9	1[d]		1.369[e]
phospholipids	775.0	6[e]		0.987[e]
DDAO	229.4	206 ± 12[e]	229 ± 16[e]	1.122[g]

[a] Feher & Okamura (1978). [b] Okamura et al. (1975). [c] Jolchine & Reiss-Husson (1975). [d] Cogdell et al. (1976). [e] This work. [f] Gouterman & Holten (1977). [g] Sardet et al. (1976).

where $\bar{v}_p$, $\bar{v}_D$, and $\bar{v}_i$ are the partial specific volumes of protein, detergent, and other bound components i, respectively. This equation can be simplified by considering a particular value of the solvent density, equal to $\bar{v}_D^{-1}$, for which the contribution of detergent vanishes, regardless of the value of δ_D (Reynolds & Tanford, 1976; le Maire et al., 1976a); the molecular weight of the protein can thus be calculated at that particular density without knowledge of the detergent binding δ_D by eq 4:

$$M^*(1 - \bar{v}^*\rho)_{\rho=\bar{v}_D^{-1}} = M_p[(1 - \bar{v}_p\rho) + \sum_i \delta_i(1 - \bar{v}_i\rho)] \quad (4)$$

In Table I the values used for the various $\bar{v}$'s and δ's are given.

Sedimentation Velocity. Sedimentation velocity experiments were performed at 60 000 rpm in a double-sector cell placed in an An-H rotor. According to the concentration of the sample, the sedimentation of reaction centers in presence of 1 mg/mL DDAO in the buffer was followed either by the Schlieren method or by recording the absorbance at one of its maxima in the visible range on photographic plates, at 4-min intervals and extrapolated to zero concentration.

The Stokes radius R_s was calculated from the sedimentation data by eq 5 (Tanford et al., 1974):

$$R_s = \frac{M^*(1 - \bar{v}^*\rho)}{6\pi\eta sN} \quad (5)$$

where η is the viscosity of the solvent, s is the sedimentation coefficient, and N is Avogadro's number.

Analytical Methods. Phosphorus content was determined by the method of Bartlett (1959) in the initial samples (after purification by DEAE-cellulose chromatography in presence of LDAO) and then in the fractions eluted from the Sepharose column, as well as in the protein fractions eluted from the combined hydroxylapatite–Sephadex column.

Protein concentration was routinely measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Standard curves were always run in the same medium, including detergent as those of the reaction center samples. Calibration of this procedure was accomplished by using the data of Steiner et al. (1974), according to which an R 26 reaction center sample (at 1 mg of protein per mL) has an absorbance at 802 nm equal to 3.13 (1-cm path length); accordingly, all Lowry determinations were divided by 1.05. The same correction was applied to the R 26 and the wild-type reaction center solutions.

Results

Binding of DDAO by Solubilized Reaction Centers. In a first series of experiments, we used gel chromatography for determining the binding of DDAO by the reaction center protein. Figure 1 shows the elution of the reaction center

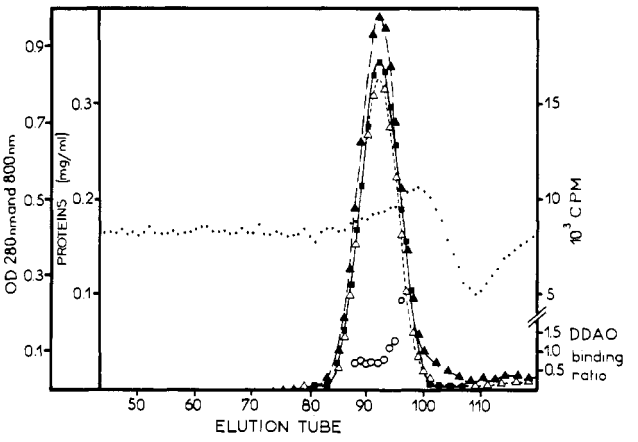

FIGURE 1: Binding of DDAO to wild-type *R. spheroides* reaction centers by gel filtration. The reaction centers in LDAO buffer solution (3.3 mg of protein per mL in a 1.5-mL total volume) were chromatographed on Sepharose 6B-CL in presence of [^{14}C]DDAO-containing buffer [DDAO (2.840 mCi/mol), 2 mg/mL]. The elution profiles of labeled detergent (···) and of reaction center protein (■) and the absorbances at 280 (▲) and 800 nm (△) are indicated. The fluctuations of DDAO binding (○) are also shown.

complex and of radiolabeled DDAO after Sepharose chromatography of the LDAO-solubilized reaction center from wild-type *R. spheroides*. Protein is eluted in a single narrow retarded peak. The concentration of DDAO increases within the protein peak; then it varies in a large peak and trough, which is due to the exchange and binding of radioactive DDAO micelles to unlabeled detergent–reaction center micelles (Hummel & Dreyer, 1962; Makino et al. 1973). Phospholipids which are only present in the wild-type reaction centers elute as strongly bound to the complex, independently of the detergent mixed micelles (not shown in the figure). Similar experiments were performed on R 26 reaction center complexes (not shown), with identical results. It is obvious from the radioactivity profile (Figure 1) that the fractions containing respectively DDAO–reaction centers and DDAO micelles are not well separated by the gel. As a result, when the binding of DDAO to the reaction center protein is determined throughout the whole protein peak, it does not stay constant (Figure 1) and rises sharply after a short plateau. In this plateau its mean values for the wild-type and the R 26 reaction centers, respectively, are 0.76 ± 0.07 g of DDAO per g of protein (standard deviation of 11 experimental points) and 0.79 ± 0.010 g of DDAO per g of protein (standard deviation for 14 points). Thus, accurate and reliable values of the DDAO binding could not be obtained by this technique.

In order to overcome this difficulty, fractions corresponding to the protein peak were isolated, and the binding of DDAO was again measured either by ion-exchange chromatography on hydroxylapatite or by a combined ion-exchange–gel filtration chromatography as described under Experimental Procedures.

In the ion-exchange chromatography, the reaction center protein was eluted sharply from hydroxylapatite as a very concentrated fraction. The mean value of the binding calculated as above (eq 1) for the wild-type reaction center, 0.66 ± 0.24 g of DDAO per g of protein (SD of 44 experimental points) is in fair agreement with the previous one but is affected by the lack of reproducibility between independent experiments, as shown by the high value of the standard deviation. Some of the variability might be due to the fact that when the protein–detergent complex is released by addition of 0.15 M phosphate, some excess detergent (previously bound to the column) is also release simultaneously and in a variable way,

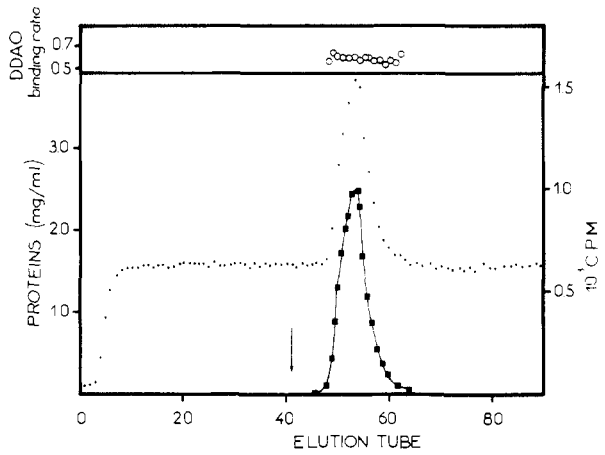

FIGURE 2: The pooled protein peak from Figure 1 was chromatographed by the combined ion-exchange–gel filtration technique and DDAO binding was determined as described under Experimental Procedures. The protein (■) and radiolabeled DDAO (···) elution profiles are shown. The arrow indicates the pulse (300 μL) of inorganic phosphate. Detergent/protein binding ratios (○) are remarkably constant across the whole protein peak.

giving an apparent binding ratio higher than the true one. To test for this possibility, in some experiments on the wild-type reaction center we determined the binding ratio again on the pooled protein fractions after elimination of phosphate by dialyzing 48 h against labeled buffer. This binding was found to be 16% lower than that before dialysis. In principle, this binding ratio should be more reliable than the one obtained by gel filtration or ion-exchange chromatography (Steinhardt & Reynolds, 1969). However, the dialysis of detergents above the critical micellar concentration might be an erratic procedure, as a true equilibrium might be very slowly or even never reached. By dialyzing [^{14}C]DDAO buffer against cold DDAO buffer both at the same DDAO concentration, we found that after 18 days there was still a 3.1% excess of radioactivity inside the dialysis bag; by measuring the radioactivity of the washed cellophan bag after dialysis and comparing it to a blank, we found that this phenomenon is presumably due to an irreversible binding of some detergent molecules to the cellophane membrane which progressively closes its pores.

Taking into account these experimental problems, the combined ion-exchange–gel filtration technique was finally used, and with this simple procedure we were able to obtain very reproducible values of the detergent–protein binding ratio, which were constant across the whole protein elution peak with very little dispersion as illustrated in Figure 2. Inorganic phosphate and a possible excess of monomeric detergent were retarded by the Sephadex moiety of the column and clearly separated from the reaction center protein (not shown in the figure). By this procedure, the following binding ratios were obtained for the wild-type reaction center protein and for the R 26 one, respectively: 0.58 ± 0.04 g of DDAO per g of protein (mean ± SD of 22 experimental points) and 0.67 ± 0.04 g of DDAO per g of protein (mean ± SD of 28 experimental points), both at a DDAO concentration of 1 mg/mL. These values were adopted for determining the molecular weight by centrifugation analysis (see below). By a few experiments performed at various DDAO concentrations between the cmc (0.5 mg/mL) (Hermann, 1962) and 2 mg/mL, we ascertained that, at the ionic strength employed and within the experimental error, the binding of DDAO to the protein remained constant.

Molecular Weight Measurements by Sedimentation Equilibrium. The equilibrium sedimentation of reaction center

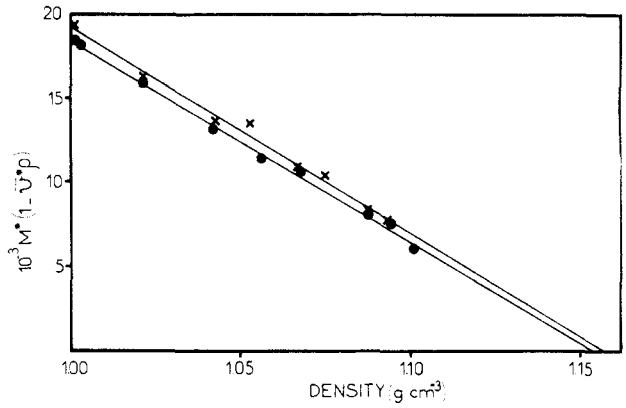

FIGURE 3: Sedimentation equilibrium of wild-type and R 26 mutant *R. spheroides* reaction center complexes in buffer containing 1 mg of DDAO per mL, 50 mM NaCl, 10 mM Tes (pH 7.5), and varying concentrations of $H_2{}^{18}O$ to give the solvent densities indicated on the abscissa. The values of $M^*(1 - \bar{v}^*\rho)$ are derived from sedimentation measurements. The line is the least-squares fit to all the data. (×) Wild-type *R. spheroides* reaction center complex; (●) R 26 mutant.

samples in the presence of 1 mg of DDAO per mL was found to be invariant during the experiment (i.e., up to 5 days). Linear equilibrium plots were observed in most of the experiments (see below), and their slopes were independent of the initial reaction center concentration (in the range 0.13–0.42 mg of protein per mL). Satisfactory recoveries (85–100%) were obtained. These findings indicate the existence of a stable state and the monodispersity of the reaction center complexes in solution. This was also corroborated by the presence of one homogeneous boundary in sedimentation velocity experiments on the same samples.

It is worth mentioning that in some preliminary experiments on the R 26 reaction center, curved equilibrium plots with a slight upward trend were observed. In sedimentation velocity experiments on these samples, we noted the presence of a second species absorbing at 365 nm, sedimenting near the main boundary, indicating the presence of pigment–detergent mixed micelles. Molecular weights calculated from these curved equilibrium plots suggested the presence of aggregated material, and the recovery was unsatisfactory. These phenomena were probably due to a partial decomposition of the reaction center, caused by the oxidant power of commercial DDAO; we avoided them by changing the source of detergent and adding catalase to the buffer solutions (see Experimental Procedures). In the following interpretation only experiments yielding linear equilibrium plots were considered.

When these experiments were performed in DDAO buffers containing various amounts of $H_2{}^{18}O$, a linear variation of the quantity $M^*(1 - \bar{v}^*\rho)$ (experimentally determined) vs. the solvent density ρ was observed (Figure 3). From such plots the partial specific volume $\bar{v}^*$ of the reaction center complex and its particle weight M^* may be directly calculated. The mean values of these parameters were determined accurately from several such plots and are given in Table II.

From M^*, the molecular weight of the protein itself, M_p, may be derived (eq 2). Indeed, the binding of detergent δ_D is experimentally determined (Table II); the other bindings δ_i corresponding to other components of the reaction centers (BChl, Bpheo, UQ, Fe, and possibly spheroidene and lipids) may be expressed as a function of number n_i, moles of component i bound per reaction center protein:

$$\delta_i = \frac{n_i M_i}{M_p} \tag{6}$$

so we may transform eq 2 into eq 7:

$$M_p = \frac{M^* - \sum n_i M_i}{1 + \delta_D} \quad (7)$$

For BChl, Bpheo, and spheroidene, the values of n_i are known from spectroscopic and chemical measurements; we adopted for UQ and Fe $n_i = 2$ and 1, respectively, as currently accepted (Table I).

On the basis of phosphorus analysis, we assumed that our preparations of the wild-type *R. spheroides* reaction center contain 6 mol of phospholipids per mol of reaction center; this number is quite smaller than the previously published value (Jolchine & Reiss-Husson, 1975) as there is a loss of lipids during the purification step used here, particularly during the DEAE-cellulose chromatography. The molecular weights of the protein (Table II), determined accurately for the wild-type and the R 26 mutant, are 81 200 ± 2100 and 78 900 ± 3600, respectively. One may furthermore conclude from these data that the reaction centers in DDAO solutions are in a monomeric state; that is, each detergent–reaction center particle only contains one set of L, M, and H subunits.

Independently of this determination, one may calculate M_p without knowledge of the value of the detergent binding δ_D by extrapolation of the equilibrium sedimentation data to a solvent density $\rho = \bar{v}_D^{-1}$ (le Maire et al., 1976a; Reynolds & Tanford, 1976). This requires, however, a knowledge of the partial specific volumes of the various components (eq 4). We calculated $\bar{v}_p$ on the basis of the amino acid composition (Steiner et al., 1974) by the classical method of Cohn & Edsall (1943) as modified by Zamyatnin (1972); the value thus obtained was 0.736 $cm^3\ g^{-1}$. For the other partial specific volumes, we assumed they were not significantly altered by association with the protein, so that experimental or calculated values relative to individual components may be used. The value of $\bar{v}_D$ given by Sardet et al. (1976) was adopted. For all other components i, $\bar{v}_i$ values were calculated on the basis of their nominal composition by using Traube's volume additivity rule (Traube, 1899; Cohn & Edsall, 1943) (Table I). For the phospholipids we adopted a mean value between partial specific volumes of phosphatidylcholine, phosphatidylglycerol, and phosphatidylethanolamine, the main lipids presumably still present (Jolchine & Reiss-Husson, 1975). We thus obtained 87 900 ± 1700 and 86 100 ± 3400 for the protein molecular weights in wild-type and R 26 reaction centers, respectively (Table II). We will analyze under Discussion the possible reasons of the discrepancy between these values and those mentioned above.

Sedimentation velocity experiments performed at various concentrations of wild-type reaction centers in a DDAO–H_2O buffer are illustrated in Figure 4; similar studies (not shown) were performed on R 26 reaction centers. It is remarquable that even at the higher concentrations (4.0 mg of protein per mL) the complexes remain monodisperse, a necessary requirement for further small-angle X-ray scattering studies. The extrapolated sedimentation coefficients $s_{20,w}$ are given in Table II. From these and sedimentation equilibrium data, the Stokes' radii of the reaction center–detergent complexes were calculated (Table II); one may note a significant difference between the values for the R 26 and the wild-type preparations.

A similar difference was also noted in gel filtration experiments, the K_{av} value relative to the wild-type reaction center complex being slightly smaller than that relative to the R 26 one. The values of R_s determined by gel filtration showed, however, a considerable deviation from those calculated from sedimentation; such an observation is common for membrane proteins in detergent solutions. Tanford & Reynolds (1976) and Nozaki et al. (1976) already pointed out that some caution is needed when measuring R_s by gel chromatography.

Table II: Particle Weight, Protein Molecular Weight, and Hydrodynamic Properties of Reaction Center Complexes

parameter	values for: wild-type Y reaction center	values for: R 26 strain reaction center
M^*	140 700 ± 2100	138 900 ± 6100
$\bar{v}^*$ (cm^3/g)	0.863 ± 0.001	0.868 ± 0.002
δ_D (g/g of protein)[a]	0.58 ± 0.04	0.67 ± 0.04
M_p[b]	81 200 ± 2100	78 900 ± 3600
M_p[c]	87 900 ± 1700	86 100 ± 3400
$s^0_{20,w}$ (S)[d]	3.57 ± 0.09	3.91 ± 0.20
R_s (A)[e]	47 ± 1	41 ± 2
R_s/R_{min}	1.28 ± 0.03	1.11 ± 0.05

[a] δ_D, mass of detergent bound per mass of protein, was determined experimentally (see the text). [b] Protein molecular weights calculated from eq 7, taking into account the experimental values of M^* and δ_D. n_i and M_i values are tabulated in Table I. [c] Protein molecular weight from the least-squares fit of the data shown in Figure 3, assuming that at $\rho = 1/\bar{v}_D$, $M^*(1 - \bar{v}^*\rho) = M_p[(1 - \bar{v}_p\rho) + \Sigma\delta_i(1 - \bar{v}_i\rho)]$ (eq 4). $\bar{v}_p$ was calculated to be 0.736 $cm^3\ g^{-1}$ from the amino acid composition (Steiner et al., 1974). [d] $s^0_{20,w}$ (S), sedimentation coefficient corrected to standard conditions, was calculated from the least-squares fit of the data shown in Figure 4 for the wild-type Y strain reaction center and from similar data (not shown) for the R 26 strain reaction center. [e] R_s, the Stokes radius from sedimentation equilibrium and velocity measurements performed at the same solvent density.

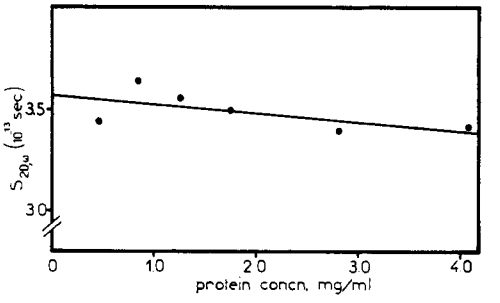

FIGURE 4: Results of sedimentation velocity experiments on wild-type *R. spheroides* reaction center complexes at various protein concentration in buffer containing 1 mg of DDAO per mL, 50 mM NaCl, and 10 mM Tes (pH 7.5). Linear extrapolation to zero concentration (solid line) was done by the least-squares fit to all the data.

Discussion

The purpose of this work was to obtain accurate physicochemical parameters for *R. spheroides* reaction centers in detergent solution: state of aggregation, weight, and size of the detergent–reaction center complex, amount of bound detergent, and molecular weight of the protein. Such a characterization seems indeed desirable, as these preparations are extensively used to study the primary electron transfer reactions taking place in the bacterial photosynthetic apparatus. The definition of isolated reaction centers is purely operational and designates the smallest complexes capable of primary photochemical activity, with unaltered kinetics as compared to the intact bacterial membrane. In *R. spheroides*, this seems to require the presence of the three polypeptide chains L, M, and H, although the role of the H subunit is still not clear and might not be essential (Feher & Okamura, 1978). LDAO (or DDAO) is considered to be the most suitable detergent both for the solubilization and purification of these reaction centers; indeed, a cationic detergent (cetyltrimethylammonium bromide), although quite effective in the solubilization, has a denaturing action (Reiss-Husson & Jolchine, 1972) whereas another nonionic detergent, Triton X-100, does not allow resolution of the reaction center from other membrane con-

stituents (Reed & Clayton, 1968). Furthermore, the stability of reaction centers purified with LDAO is amazingly high. Thus, the choice of LDAO (and DDAO) in this work was quite natural and mandatory; nevertheless, DDAO is not well suited for sedimentation studies as its specific volume is quite high, as we stressed below.

The first unambiguous conclusion is that, at least when purified as described, at the ionic strengths and within the concentration ranges studied (DDAO: from the cmc to 2 mg/mL), the reaction center–detergent complexes are in a stable and monodisperse state. This state is monomeric; that is, only one reaction center is present per particle.

Equilibrium sedimentation in DDAO buffers containing various H_2O–$H_2{}^{18}O$ mixtures thus led to a straightforward determination of the particle partial specific volume $\bar{v}^*$ and of the particle weight M^*. The values of the particle weights M^* relevant to reaction center complexes of both strains are very similar (140 700 ± 2100 for the wild type; 138 900 ± 6100 for R 26); the slight difference between the two values may result from the nonidentical composition, stemming from the different numbers of DDAO, spheroidene, and lipid molecules bound per reaction center particle. The values of $\bar{v}^*$ obtained in this work are 0.863 ± 0.001 $cm^3\ g^{-1}$ for the wild-type reaction center–detergent complex and 0.868 ± 0.002 $cm^3\ g^{-1}$ for the R 26 one. Independent measurements of $\bar{v}^*$ of protein–detergent complexes are exceedingly difficult to obtain by densimetry (Tanford et al., 1974). However, our experimental value of $\bar{v}^*$ obtained from sedimentation equilibrium may be compared with the values calculated assuming additivity of partial specific volumes of individual components of the reaction centers given in Table I (some of them being already calculated values) and adopting for δ_D the experimental value determined by chromatography (eq 8):

$$\bar{v}^* = \frac{\bar{v}_p + \delta_D \bar{v}_D + \sum \delta_i \bar{v}_i}{1 + \delta_D + \sum \delta_i} \qquad (8)$$

The $\bar{v}^*$ values thus calculated are 0.881 ± 0.005 $cm^3\ g^{-1}$ for the wild-type reaction center and 0.888 ± 0.005 $cm^3\ g^{-1}$ for the R 26 one; they differ by 2% from the experimental values. This might be due to the simplyfing assumption of eq 8 regarding the linear additivity. On the other hand, it might also indicate that the choice of some of the parameters in eq 8 was incorrect. The major contributions to $\bar{v}^*$ come from $\bar{v}_p$, $\bar{v}_D$, and δ_D. The value adopted for $\bar{v}_p$ is based on assumptions commonly used for protein; that adopted for $\bar{v}_D$, which is the one given by Sardet et al. (1976), has not been verified and can not be checked as there is, to our knowledge, no other determination of specific volumes of alkylamine oxides. The detergent binding, δ_D, was measured as accurately as possible in order to derive M_p from M^* without any assumption on the additivity of partial specific volumes.

This precise measure of δ_D was unexpectedly difficult and needed the comparison of data obtained by different procedures. Data obtained by the gel filtration on Sepharose 6B-CL according to Hummel & Dreyer (1962) suffered from the fact that the reaction center complex containing fractions were not well separated from detergent micelles. This resulted in the fact that the binding ratio obtained were neither constant across the whole elution peak nor quite reproducible among different experiments. Similar difficulties were experienced when ion-exchange chromatography on hydroxylapatite was used instead of gel filtration. Here also, the binding ratios appeared to be too large, presumably owing to a contribution by detergent micelles eluted together with the detergent–protein complex. Indeed, when a dialysis was performed for eliminating the excess of DDAO micelles together with the phosphate ions, we observed a significant decrease in the binding ratio values. But, as shown under Results, dialysis did not seem to be a satisfactory technique. We adopted finally as the most reproducible method the combined ion-exchange–gel filtration technique (Figure 2), which in a short experiment allowed us to determine the binding ratios of the reaction centers free of phosphate (Table II). One may observe that the numer of DDAO molecules bound per reaction center is slightly higher in the R 26 complex; it can be speculated that in the wild type, the presence of phospholipids fulfills for a part the requirements of covering the hydrophobic surface of the protein. In both cases, this number of bound DDAO molecules corresponds to more than two DDAO micelles per protein molecule (Hermann, 1962); this high amount of detergent required to keep the reaction centers in monomeric solution is another evidence for the very pronounced hydrophobic character of the protein, which belongs to the class of integral membrane proteins (Steiner et al., 1974). Ackerson, as quoted by Feher & Okamura (1978), found by the dialysis method a binding ratio of 0.47 ± 0.06 g of LDAO per g of protein in R 26 reaction centers. This value (besides being relative to commercial LDAO which is composed of a mixture of fatty acids) refers to solutions containing 0.25 mg of LDAO per mL, that is, below the critical micellar concentration. This might explain a binding ratio smaller than the one we found for DDAO, as this parameter, as well as the state of aggregation of the reaction centers, may well vary strongly below the critical micellar concentration (Tanford & Reynolds, 1976).

One may argue that the binding of detergent was not measured in the same experimental conditions as those prevailing in an sedimentation equilibrium experiment. Indeed, in the last case, there is a gradient of concentration of DDAO throughout the cell, the detergent micelles tending to float because their density is lower than that of water. Thus, the protein is in equilibrium with a local concentration of DDAO probably lower than the mean one and difficult to estimate. This effect should be more and more pronounced the higher the solvent density. However, we feel confident that the DDAO binding is not appreciably modified by this DDAO gradient. First, we did not observe a dependence of δ_D (measured by chromatography) upon the DDAO concentration, at least above the critical micellar concentration. Second, the linearity of the plot $M^*(1 - \bar{v}^*\rho)$ vs. ρ would be difficult to explain if δ_D were affected by the DDAO gradient and thus were dependent on ρ. Thus, it seems reasonable to adopt for the detergent binding at sedimentation equilibrium the values determined by chromatography experiments. Because of the difficulties encountered during the latter, however, the error on δ_D is unlikely to be smaller than 11–12%.

These possible inaccuracies in either the various partial specific volumes or in the detergent binding (or in both) should be kept in mind when the molecular weights of the protein are considered. Taking into account the experimentally determined δ_D, and the contribution of several constituents other than polypeptide chains (Table I), we derived from M^* values of 81 200 ± 2100 and 78 900 ± 3600 for the wild-type and the R 26 reaction center proteins, respectively. These values are highly dependent on the detergent binding δ_D: a 5% variation in δ_D would result in a difference of ~1700 in M_p.

On the other hand, when M_p was determined by extrapolation of the data to $\rho = \bar{v}_D{}^{-1}$, higher values were obtained (87 900 ± 1700 and 86 100 ± 3400 for wild-type and R 26 reaction center proteins), i.e., 87 300 ± 2000. The inaccuracy

of the method comes from the long extrapolation (to an hypothetical density of 0.891 g cm^{-3}) which is needed to blank out the DDAO (Reynolds & Tanford, 1976; le Maire et al., 1976a). In contrast, other detergents such as, for instance, lubrol have a partial specific volume slightly below 1.0 (for lubrol WX, $\bar{v}$ = 0.929 cm^3 g^{-1}) so that the density needed to blank them out is within experimental reach with $H_2{}^{18}O$ or D_2O (Tanford & Reynolds, 1976). However, the accuracy of the data (Figure 3) seems to allow for such an extrapolation, and, since the accuracy in measuring M_p heavily depends on the amount of detergent bound, one is tempted to put more faith into a result which does not rely upon any detergent/protein binding ratio. Of course, the extrapolation is very sensitive to the value adopted for $\bar{v}_D$, and the determination of M_p depends on the values adopted for the other specific volumes $\bar{v}_p$ and $\bar{v}_i$. If we adopt the extrapolated values of M_p (87 900 and 86 100) for each reaction center, we deduce the detergent binding ratios by eq 7. These values are equal to 0.46 and 0.53 g of DDAO per g of protein for the wild-type and the R 26 reaction centers, respectively. These values are ~20% lower than the measured detergent binding, which, considering the problems we uncountered in measuring the latter, is a fair agreement.

It may be concluded that, because of the numerous parameters involved in the determination of M_p, a choice between the two determinations is difficult. We propose that the accurate value of M_p is within the range 80 000–87 300; therefore, we suggest the mean value 84 000.

According to Tanford et al. (1974), hydrodynamic measurements such as that of the Stokes' radius can distinguish between globular particles (that is, compact and sparingly solvated) and elongated ones; for this purpose one first calculates the minimum possible radius of the particle, which would be the radius of a perfect sphere composed of protein, detergent, and eventually bound lipid, but not bound water; i.e.

$$\frac{4\pi}{3}NR_{min}{}^3 = M_p(\bar{v}_p + \delta_D\bar{v}_D + \sum_i \delta_i\bar{v}_i) \qquad (9)$$

Since the particle in reality should contain some bound water and may not be perfectly spherical, an R_s value larger than R_m may be expected. As demonstrated by studies of globular native proteins or globular detergent micelles, a ratio R_s/R_m smaller or equal to 1.25 (Tanford, 1961) would be indicative of a globular shape. Our measurements of this ratio (Table II) allow us to conclude that the reaction center–DDAO complexes fall into this category; it might be that the wild-type reaction center particles, for which this ratio is at its upper limit, are slightly different from the R 26 one, either in compacity, in solvation, or in anisodiametry. It is of interest to mention that so far most membrane proteins which have been characterized in detergent solution have a higher value of R_s/R_{min} (between 1.5 and 1.6) (Tanford & Reynolds, 1976; le Maire et al., 1976b).

A more detailed analysis of the shape of the protein and respective location of detergent and protein moieties will be the subject of a small-angle X-ray scattering study which is now in progress. In this respect, the use of DDAO which has a partial specific volume very different of the protein moiety is ideal.

Soon after this article was submitted the work of Vadeboncoeur et al. (1979) was published. In the presence of Triton X-100, the "molecular weight" determined by Vadeboncoeur et al. for wild-type *R. spheroides* reaction centers is 87 000, a figure apparently in good agreement with our results. It should be noticed, however, that the interpretation of the sedimentation equilibrium experiments does not take into account the contribution of chemical compounds other than protein and detergent; yet pigments and lipids represent a mass of 12 000 per protein moiety, to be substracted from their value of 87 000. Futhermore, the accuracy of the detergent binding is not specified; in the case of *Rhodospirillum rubrum* reaction centers, for which the experiments are described in more detail, this accuracy seems fairly low.

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Circular Dichroism and Polarized Fluorescence Characteristics of Blue-Green Algal Allophycocyanins†

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ABSTRACT: Allophycocyanin (APC), the terminal pigment in the phycobiliprotein transfer sequence, isolated from dissociated phycobilisomes of *Nostoc* sp., was fractionated on calcium phosphate columns into four spectral forms: APC I, II, III, and B. These forms had distinctive isoelectric points of 5.15, 4.68, 4.82, and 4.98, respectively. The APC forms differed in their secondary structure as suggested by the varying percentages of their α helix and β-pleated sheets. APC II and III are short-emitting forms with a fluorescence maximum at 660 nm, while APC I and B are long-emitting forms with a maximum at 681 nm (at 24 °C in 0.1 M phosphate buffer). The maximum of APC I and B at −196 °C in 0.1 M phosphate shifted to 685 nm, and in 60% potassium glycerol phosphate and 20% glycerol shifted to 688 nm. Fluorescence polarization spectra suggest that there are at least two groups of chromophores responsible for the absorption of APC I and similarly of APC B. In APC II and III, the fluorescence was mostly depolarized. Circular dichroism (CD) revealed extensive positive and negative ellipticity band multiplicities in the chromophore absorption region of APC I and B, but not in APC II and III. Two main CD extrema in APC B, a negative band (668 nm) and a positive band (680 nm), are probably the result of exciton coupling of phycocyanobilin chromophores absorbing at longer wavelength. In APC I three different peaks are revealed in the absorption spectrum and four ellipticity bands in the CD spectrum at −196 °C. These can best be explained as being due to the combined interactions of the chromophore with the protein and exciton coupling between chromophores. The CD signal of APC I was shifted by disruption of strongly coupled chromophore–chromophore interaction with a chaotropic agent treatment (LiCl). It was almost abolished by disrupting both chromophore–protein and chromophore–chromophore interactions upon denaturation with sodium dodecyl sulfate ($NaDodSO_4$). Exciton interaction implies a unique structural feature where some of the chromophores in APC I and also in APC B are at a close range to each other and, therefore, most likely at the contact region between subunits. Energy transfer in some of the chromophores of the far-emitting forms is by a delocalized exciton mechanism in contrast to the short-emitting forms. Both APC I and B display an absorption peak at 680 nm (−196 °C), which is considered to be the emitting state in these long-emitting forms. APC I and B fluorescence maxima were identical with intact phycobilisomes, thus suggesting that both are final emitters of phycobilisomes. The exciton mechanism provides the bridging pigments, APC I and B, with more efficient energy transfer to chlorophyll due to increased spectral overlap between the APC fluorescence and chlorophyll absorption in vivo.

In the blue-green alga *Nostoc* sp., as in other red and blue-green algae, the light harvesting accessory pigments of photosynthesis are water-soluble phycobiliproteins contained within large aggregates called phycobilisomes that are attached to the photosynthetic membranes. In vivo, most of the excitation energy of the phycobiliproteins is transferred via allophycocyanin (APC)[1] to chlorophyll *a* with high efficiency (reviewed in Gantt, 1975). The phycocyanobilin chromophores in APC are covalently attached to the apoproteins (review by Bogorad, 1975). Allophycocyanins can be classified into two main groups according to their fluorescence wavelength emission spectra: those with a short emission at ca. 660 nm and those with a long emission at ca. 680 nm. A short-emitting form with an absorption peak at 650 nm has been isolated in many laboratories. It is a common form that is relatively easily obtainable and has been extensively studied (Bogorad, 1975; Brown et al., 1975; Brown & Troxler, 1977; Cohen-Bazire et

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[1] Abbreviations used: APC, allophycocyanin; CD, circular dichroism; $NaDodSO_4$, sodium dodecyl sulfate.