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FUNCTIONAL BACTERIOCHLOROPHYLL-PROTEIN COMPLEXES FROM CHROMATOPHORES OF *RHODOPSEUDOMONAS SPHEROIDES* STRAIN R-26*

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SUMMARY

1. The chromatophore membrane system of the blue-green mutant strain R-26 of *Rhodopseudomonas spheroides* has been fractionated by Triton X-100 into two separable and distinct kinds of particles, each containing one of the two functional forms of bacteriochlorophyll. The smaller reaction center particles contain only the reaction center bacteriochlorophyll, P870, and the associated P800. The relative ratios of electron transfer components in these preparations are about the same as in chromatophores. The reaction centers contain $4.4 \cdot 10^5$ g of protein per mole of P870 and appear predominantly as small particles in electron micrographs.

2. By contrast, the particles of the light-harvesting component contain the light-harvesting bacteriochlorophyll and are nearly devoid of reaction centers. Micrographs show particles as large smooth discs. The preparations contain $2.5 \cdot 10^4$ g of protein per mole of bacteriochlorophyll. These large light-harvesting component particles are further fractionated by sodium dodecyl sulfate into sodium dodecyl sulfate fragments, small bacteriochlorophyll proteins. The chemical and physical properties of these sub-chromatophore particles are discussed with special regard to the organization of the chromatophore membrane.

INTRODUCTION

Several physical techniques have been used to study the structure of the chromatophore membranes from the purple photosynthetic bacteria. Electron microscopic investigations have indicated that these are single membranes of approx. 80 nm thickness[1,2] and the major portion of the lipids are believed to be located within the membrane structure[3]. Measurements of low angle X-ray scattering have indicated that the arrangement of lipids and proteins within these membranes is highly asymmetric[2,4]. These studies have provided much information concerning the organization of the lipoproteins in the membranes but are unable to localize the photosynthetic pigments.

The functional association of the two forms of bacteriochlorophyll in the chromatophore membranes has been formulated from measurements correlating fluor-

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escence of the light-harvesting bacteriochlorophyll with absorbance of the reaction center bacteriochlorophyll[5-7]. The majority of the bacteriochlorophyll serves as a pool of light-harvesting pigment to absorb light quanta and to transfer this energy to reaction centers distributed throughout this pool. The reaction centers compete for quantum energy and utilize it to initiate photochemistry. The bacteriochlorophyll of the reaction centers, P870, is a small portion of the total bacteriochlorophyll which becomes oxidized in the primary photochemical act.

Another experimental approach to the molecular organization within the chromatophore membrane system is to chemically fractionate the membranes into particles and to relate their chemical and physical properties to those of the intact structures. In this report we will present results of such studies on chromatophores from the blue-green mutant strain R-26 of *Rhodopseudomonas spheroides*. These chromatophores afford several advantages for fractionation studies. The pigment composition is simplified by the presence of only a single light-harvesting bacteriochlorophyll absorption band and by the absence of carotenoids. The ratio of light-harvesting to reaction center bacteriochlorophyll is favorably low in these membranes. As has been reported briefly, these membranes are fractionated by Triton X-100 into two separate subchromatophore particles, each with a different form of bacteriochlorophyll[8]. The isolation and composition of the smaller reaction center complex particles containing the reaction center P870 has been reported in detail[9].

In the present investigation, we have sought more details of the relation between the chemical and physical properties of the two subchromatophore particles and those of the intact chromatophore membrane system. An additional procedure was developed for fractionating the large particles containing the light-harvesting bacteriochlorophyll into small bacteriochlorophyll proteins. The compositions of reaction center complex and the light-harvesting bacteriochlorophyll protein preparations were compared with the composition of the intact chromatophores. Attempts were also made to relate the physical properties of the particles revealed by electron microscopic techniques.

MATERIALS AND METHODS

Bacteriochlorophyll concentrations were calculated from the absorbance of their near infrared bands measured at 25° with a Cary 14R recording spectrophotometer. The extinction coefficient of 127 $mM^{-1} \cdot cm^{-1}$ (see ref. 10) was used for the light-harvesting bacteriochlorophyll. Concentrations of the reaction center P870 were calculated from absorption spectra using the extinction coefficient of 113 $mM^{-1} \cdot cm^{-1}$ (see ref. 10) or from the measurements of the reversible light-induced absorbance decrease at 865 nm using the differential extinction coefficient of 93 $mM^{-1} \cdot cm^{-1}$ (see ref. 11). Light-induced absorbance changes were measured with an 865 nm interference filter (Baird Atomic, 10 nm band width) in the measuring beam and right-angle actinic illumination from a tungsten-iodide lamp (Sylvania Sun Gun) passed through a water filter and an 800 nm interference filter.

Cytochromes were identified from difference spectra of their reduced minus oxidized forms (with and without sodium dithionite). Concentrations were calculated from an assumed differential extinction coefficient of 20 $mM^{-1} \cdot cm^{-1}$ for the alpha bands. Ubiquinone was identified from difference spectra of reduced minus oxidized

samples and measured by the extraction procedure of PUMPHREY AND REDFEARN[12].

Total iron and copper were measured by ashing the samples and complexing the iron with bathophenanthroline[13] or the copper with bathocuproin[14]. Copper was also measured in ashed samples by atomic absorption techniques. Dry weights were determined by bringing samples of known bacteriochlorophyll concentrations to constant weight under reduced pressure over phosphorus pentoxide at 60°. Lipid-extractable material was determined as the loss in dry weight following five extractions with chloroform–methanol (2:1, v/v). Protein was measured by the method of LOWRY *et al.*[15].

For electron microscopy, dilute suspensions of subchromatophore particles were placed directly onto carbon coated grids and washed *in situ* with 1 or 2 % solutions of phosphotungstic acid, titrated to pH 6.0 with potassium hydroxide. Alternatively, the suspension was spread as a monolayer from the point of a needle onto a drop of phosphotungstic acid and then picked up on a grid. Bovine serum albumin (0.05 %) was occasionally incorporated into the phosphotungstic acid to help spread it. In all cases, control grids were examined to exclude the possibility that the image observed was other than the membrane fraction.

R. spheroides, strain R-26, was grown according to the method of SISTROM AND CLAYTON[16], rinsed with 0.1 M Tris–HCl buffer (pH 7.5) and stored as a frozen paste at —10°. All remaining steps were carried out at 0–10° in 0.01 M Tris–HCl buffer (pH 7.5). The bacterial cells were broken with a Aminco French pressure cell at 20000 lb/inch^2. The chromatophore fraction was isolated from the broken cell suspension as the fraction which sedimented between 20000 × *g* for 15 min and 240000 × *g* for 60 min. The pellets were suspended in buffer to a bacteriochlorophyll concentration of 0.71 mM.

Small amounts of ribosomal and cell wall material were separated from the chromatophores by an additional sedimentation on a linear sucrose density gradient. 4 ml of the chromatophore fraction were layered onto 34 ml gradients formed linearly from 0.5 to 1.5 M sucrose in 38 ml Spinco centrifuge tubes. The samples were centrifuged for 5 h at 95000 × *g*. Two separate pigmented bands formed in each tube. The lower lightly pigmented bands were discarded. The upper heavily pigmented chromatophore bands were combined, diluted with approximately two volumes of buffer, and sedimented by centrifugation for 60 min at 240000 × *g*. These pellets of purified and washed chromatophores were suspended in buffer and diluted until the absorbance at 862 nm was 50 (0.39 mM bacteriochlorophyll).

Chromatophores were treated with two different Triton X-100 concentrations to obtain either maximal yield or maximal integrity of the reacton center complex particles. The lower 1 % Triton avoided alteration of the cytochrome components of the complex and 2.3 % detergent yielded maximal recovery of P870. The appropriate volume of a 10 % Triton X-100 solution (0.11 or 0.30 volume) was added slowly to a stirring chromatophore suspension. Portions of the mixture, 4 ml, were layered onto discontinuous density gradients formed from 4 ml layers of 0.5 and 1.0 M sucrose in 13.5 ml Spinco centrifuge tubes. After centrifuging the samples for 90 min at 240000 × *g*, the reaction centers remained in the supernatant layers above the sucrose and were pooled. The larger particles containing the light-harvesting bacteriochlorophyll formed bands at the interface between the two sucrose layers.

The supernatant fractions were combined, dialyzed exhaustively against buffer and concentrated by ultrafiltration on an Amicon XM-100 membrane. 5 ml portions

of the concentrated solution were chromatographed on a 3.4 cm × 45 cm Agarose A5m column (BioRad, 50–100 mesh) at a flow rate of 40 ml per h. Fractions containing P870 eluted as a major peak near 180 ml (1.1 void volume) and those fractions which exhibited a ratio of absorbance at 803 nm to that at 757 nm of greater than 1.4 were combined. This purified reaction center preparation was concentrated by ultrafiltration when necessary.

The large particles containing the light-harvesting bacteriochlorophyll (light-harvesting component) were isolated following treatment of chromatophores with 2.3 % Triton X-100. The lower bands from sucrose gradients were combined, diluted with approx. 10 volumes of buffer and sedimented by centrifugation for 60 min at 240000 × *g*. These pellets of the light-harvesting component were suspended in buffer and diluted until the absorbance at 850 nm was 38.

The light-harvesting component particles were treated with the anionic detergent, sodium dodecyl sulfate, to dissociate them into small bacteriochlorophyll–protein particles (sodium dodecyl sulfate fragments). The samples were maintained in dim light throughout the procedure. Sodium dodecyl sulfate (5 %, w/v), 0.2 ml, was added slowly to 1 ml of the concentrated suspension of the light-harvesting component. The mixture was stirred for an additional 5 min and applied to a 2.2 cm × 40 cm Agarose A5om column (BioRad, 50–100 mesh). The column was washed with buffer at a flow rate of 10 ml per h and fractions of 2.5 ml were collected. The absorbance at 850 nm was measured in the fractions. The maximum absorbance occurred after collecting approx. 86 ml. Those fractions in which the absorbance at 850 nm exceeded 0.4 were combined as the sodium dodecyl sulfate fragment preparation. This suspension was concentrated by ultrafiltration when necessary. The final concentration was kept below an absorbance of 5.0 to avoid reaggregation of these particles.

RESULTS

The purified chromatophores obtained by sedimenting the chromatophore fraction on the linear sucrose density gradient provided suitable starting material for fractionation studies. In these preparations, the ratio of ultraviolet absorbance at 276 nm to bacteriochlorophyll absorbance at 862 nm was near 0.8 (Fig. 1). The purified chromatophores were eluted from the Agarose A5om column in a single symmetrical peak and were also quite uniform when viewed by electron microscopy (Fig. 3).

Typical recoveries and compositions of the particles obtained by detergent fractionation of chromatophores from *R. spheroides* stain R-26 are listed in Table I. The distribution of the reaction center P870 between the two subchromatophore fractions was strongly dependent on the final Triton X-100 concentration added to the chromatophore suspension. The higher 2.3 % Triton X-100 yielded the maximum recovery of P870 in the purified reaction center complex and the minimum amount of P870 remaining with the larger particles in the light-harvesting component preparation. Some bacteriochlorophyll was solubilized and the preferential loss of the light-harvesting form caused the relative amount of P870 in the light-harvesting component preparation to appear misleadingly large.

Absorption spectra of the reaction center complex preparations isolated with 2.3 % Triton X-100 are shown in Fig. 2. After allowing the sample to remain in the dark, the absorption band of P870 appeared with a maximum at 865 nm (solid line).

This absorption band was completely and reversibly bleached by illuminating the sample with 800 nm actinic light (dashed line). The absorption spectra of reaction center preparations isolated using 1 % Triton X-100 differed only slightly from those in Fig. 2. The higher cytochrome content in these more intact reaction center preparations (Table II) was accompanied by increased absorbance near 400 nm with the appearance of a distinct absorption maximum near 405 nm.

Typical analyses of the relative chemical compositions of both chromatophore and reaction center complex preparations are listed in Table II. The chromatophores were isolated in the low ionic strength buffer to remove loosely associated electron transfer components before exposing these "washed" membranes to detergents. Reaction centers were then isolated from these chromatophores using the lower 1 %

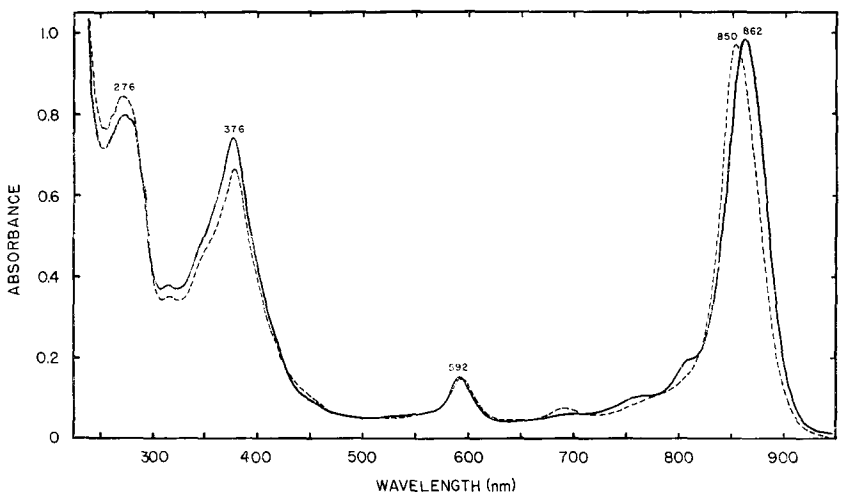

Fig. 1. Absorbance spectra of particle preparations from strain R-26 of *R. spheroides* containing the light-harvesting bacteriochlorophyll. The samples are chromatophores (——) and light-harvesting component (– – – –) in 0.01 M Tris–HCl buffer, pH 7.5.

TABLE I

FRACTIONATION OF THE CHROMATOPHORE MEMBRANE SYSTEM OF *R. spheroides* STRAIN R-26

The recoveries are the yield of the major bacteriochlorophyll (BChl) form in the fraction obtained from chromatophores: reaction center P870 (RC) or light-harvesting (LH). The reaction center complex was isolated using 2.3 % Triton X-100. Compositions are relative to the content of this bacteriochlorophyll form.

Fraction	*BChl recovery* (%)	*Composition*		
		BChl (moles LH/mole RC)	*Protein (g/mole BChl* × 10^{-5})	*Total lipid*
Chromatophore	100	32	0.31	0.16
Reaction center complex	65	0	4.4	2.0
Light-harvesting component	50	188	0.25	0.02
Sodium dodecyl sulfate fragment	34	> 500	0.27	—

Triton X-100 to avoid alteration of the electron transfer components of the complex at the expense of a lower overall recovery. It is evident from these analyses that the reaction center complex preparations contained the same relative ratios of electron transfer components as the chromatophores but without the complement of light-harvesting bacteriochlorophyll, some protein, and lipid.

Electron micrographs of reaction center preparations (Fig. 4) showed particles primarily as flakes 12 nm in diameter. This particle size was consistent with the mini-

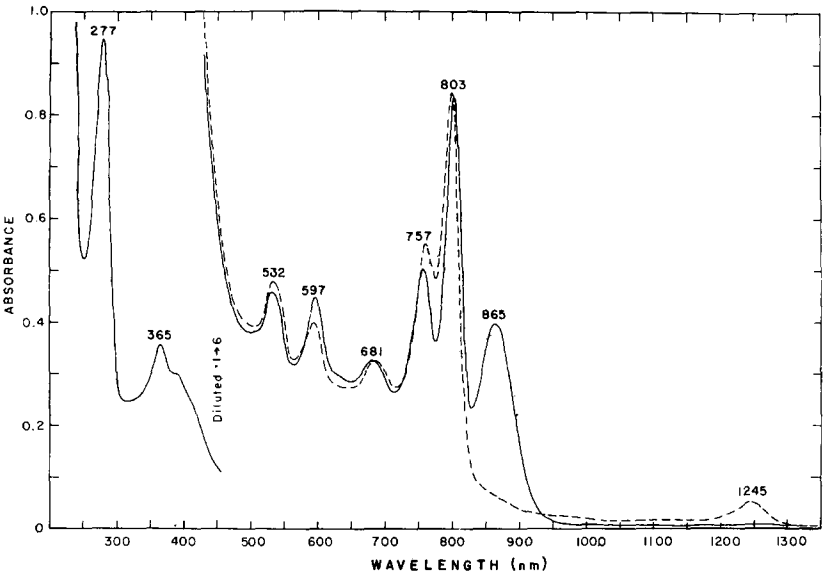

Fig. 2. Absorbance spectra of the purified reaction center complex in 0.01 M Tris–HCl buffer, pH 7.5 .The reaction center complex was isolated using 2.3% Triton X-100. Spectra were measured in the "IR-1" mode of a Cary model 14R recording spectrophotometer during illumination with only the monochromatic measuring beam (——) and during cross-illumination with 800 nm actinic light – – – –) sufficient to produce maximal absorbance decrease at 865 nm.

TABLE II

COMPOSITION OF CHROMATOPHORE AND REACTION CENTER P870 COMPLEX*

Components	*Preparation*		
	Chromatophore (*Amount/mole of P870*)	*Reaction center P870 complex*	
P870	1.0	1.0	mole
B870	32	0	mole
Cytochrome b_{562}	1.9	1.8	moles
Cytochrome c_{552}	1	1	mole
Total iron	14	16	moles
Copper	0.3	0	mole
Ubiquinone	12	13	moles
Total dry wt.	$15 \cdot 10^5$	$6.4 \cdot 10^5$	g
Lipid-extractable dry wt.	$5.0 \cdot 10^5$	$2.0 \cdot 10^5$	g

*Prepared with 1% Triton X-100.

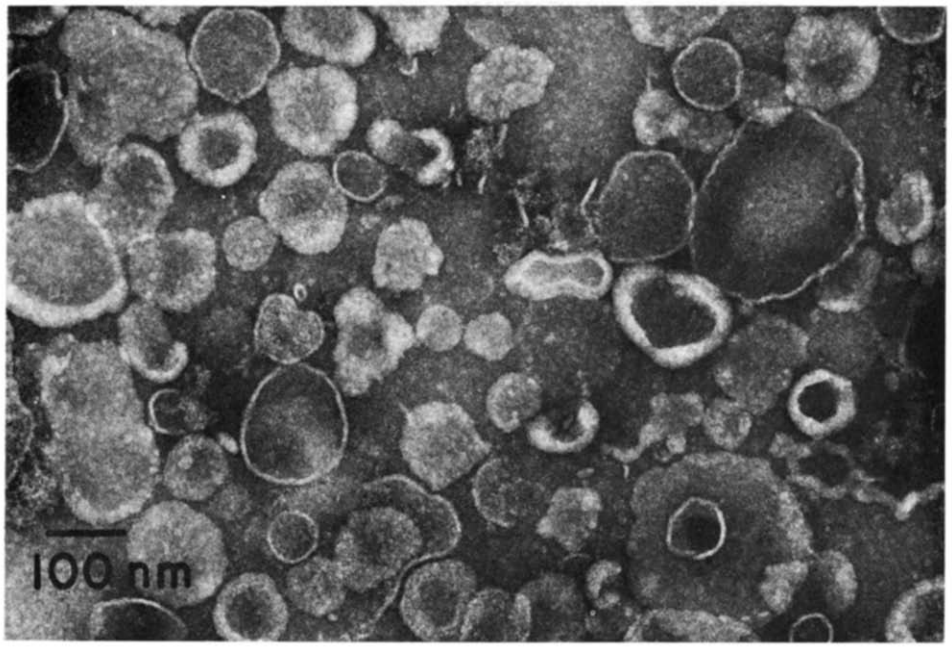

Fig. 3. Purified chromatophores. Sample was negatively stained on a grid by washing with 2% potassium phosphotungstate, pH 6.0. These vesicles are quite uniform in size and texture. The surface has a tight and subtly defined substructure.

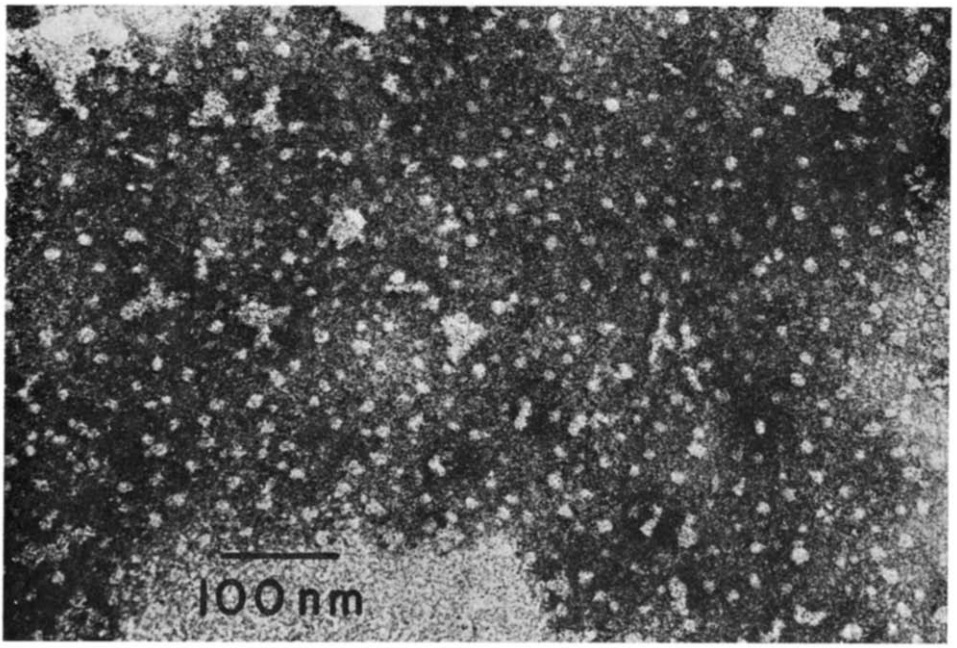

Fig. 4. Reaction centers prepared with 1% Triton X-100. Particles were negatively stained by spreading as a monolayer on a droplet of 2% potassium phosphotungstate. These flakes are typically 12 nm in diameter and aggregate extensively.

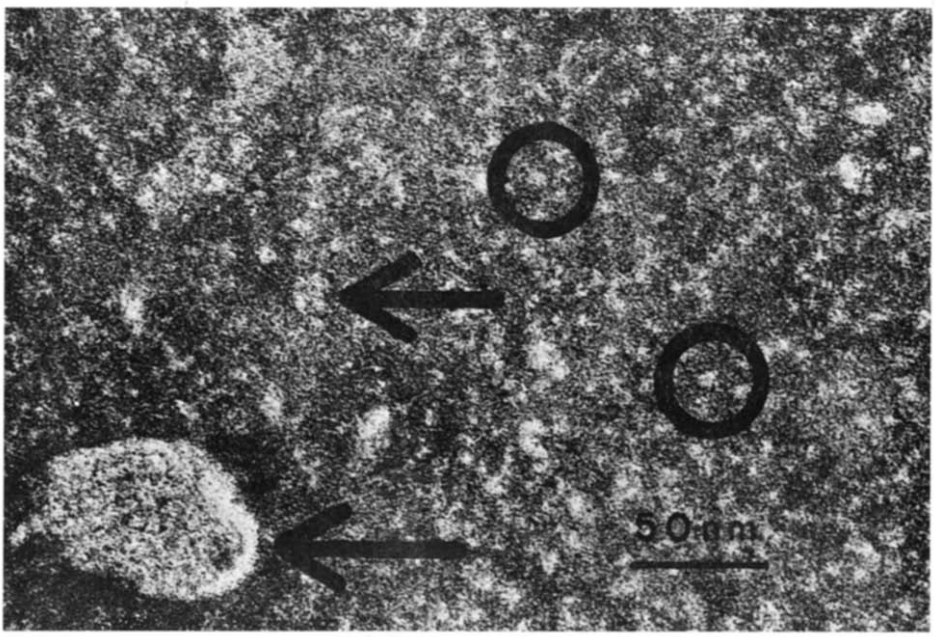

Fig. 5. Light-harvesting component. The sample was negatively stained on a grid by first washing with 2 % potassium phosphotungstate, 0.05 % bovine serum albumin, and then with 2 % potassium phosphotungstate. The vesicles resemble chromatophores in size and shape but appear thinner and smoother.

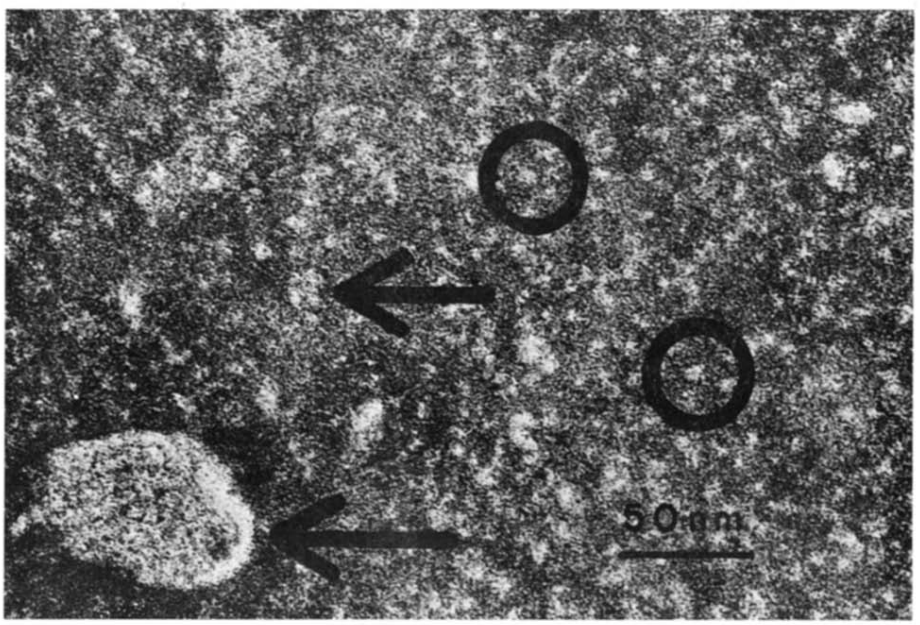

Fig. 6. Sodium dodecyl sulfate fragments. Particles were negatively stained by washing on a grid, first with 1 % potassium phosphotungstate, 0.05 %, bovine serum albumin, then with 1 % potassium phosphotungstate. These are small particles (5 nm, see circles) which readily aggregate to form small groups or larger sheets (see arrows).

mal particle weight calculated from dry weight measurements (Table II). The preparations had a tendency to aggregate and larger particles were more prevalent in stored preparations. The relative chemical composition of the aggregates removed by centrifugation for 60 min at 150000 × *g* did not differ significantly from that of the reaction centers remaining in the supernatant.

Light-harvesting component preparations isolated by 2.3 % Triton X-100 contained little lipid besides bacteriochlorophyll (Table I). The absorption spectrum of these preparations differed in two significant respects from that of the initial chromatophores (Fig. 1). The 803 nm absorption band of the reaction center component, P800, was absent and the long-wave absorption band of the light-harvesting bacteriochlorophyll shifted 12 nm toward shorter wavelength. Electron micrographs of light-harvesting component preparations (Fig. 5) showed the particles as large thin sheets.

The absorption spectrum of the sodium dodecyl sulfate fragment preparations was indistinguishable from that of the light-harvesting component preparations (Fig. 1, dashed line). The bacteriochlorophyll to protein ratios were also very similar (Table I). The sodium dodecyl sulfate fragment preparations eluted from the Agarose A50m column as a single peak with apparent particle size much smaller than the light-harvesting component and electron micrographs of negatively stained preparations (Fig. 6) showed particles primarily as small discs of approx. 5 nm diameter. Some aggregates were also apparent.

DISCUSSION

The chromatophore membrane from strain R-26 of *R. spheroides* is fractionated asymmetrically by the nonionic detergent Triton X-100. The two functional forms of bacteriochlorophyll are obtained on separate and distinct particles. The small reaction center complex particles contain the reaction center bacteriochlorophyll, P870, with the associated P800 and other electron transfer compounds and the large light-harvesting component particles contain the light-harvesting bacteriochlorophyll (Table I).

The reaction center particles are electron transfer complexes containing P870 and other electron transfer compounds. The relative ratios of electron transfer compounds in the reaction center preparations are essentially identical to that of the intact chromatophores which each contain many reaction centers (Table II). These analyses suggest that the reaction center complex is a repeating unit of photosynthetic electron transfer in the chromatophore membrane system.

The light-harvesting component particles contain essentially only light-harvesting bacteriochlorophyll and protein (Table I) and thus account for the remaining chromatophore components not present in the reaction center complex preparations (Table II). These particles appear as large sheets when viewed in the electron microscope (Fig. 5) similar to the large particles obtained by GARCIA *et al.* from chromatophores of *Chromatium*[18] or *R. rubrum*[19] also using Triton X-100. Sodium dodecyl sulfate-treatment fractionated these sheets into the much smaller sodium dodecyl sulfate fragments (Fig. 6). The sodium dodecyl sulfate fragment preparations do form large aggregates when concentrated in the absence of detergent.

The size of the sodium dodecyl sulfate fragment particles (5 nm) is similar to that of the chlorophyll–protein complexes obtained from a variety of other photosynthetic systems by THORNBER AND OLSON[20–22] also using sodium dodecyl sulfate. The

size of the protein particles in the plane of the thylakoid membrane was also concluded to be near 5 nm by both MENKE[2] and LANGRIDGE *et al.*[4] from their X-ray scattering measurements.

The infrared absorption maxima of bacteriochlorophyll in chromatophores has previously been shown to occur at considerably longer wavelengths than bacteriochlorophyll in organic solvents[17]. The specialized environment giving rise to this red shift has been variously attributed to interaction of the bacteriochlorophyll with other bacteriochlorophyll molecules, proteins, lipids, or for the reaction center P870, association with the primary electron acceptor and the two P800 molecules. The absorption spectrum of the reaction center bacteriochlorophyll P870 and the associated P800 is not altered during isolation of the reaction center complex and the absorption maximum of the light-harvesting bacteriochlorophyll only shifts from 862 nm in chromatophores to 850 nm in the light-harvesting component. This small shift of 12 nm accompanies removal of the majority of the reaction centers and non-bacteriochlorophyll lipid from the particles containing the light-harvesting bacteriochlorophyll and dissociation of these large aggregates with sodium dodecyl sulfate has no additional effect. However, addition of the proteolytic enzyme, α-chymotrypsin (Sigma, Type II), to any of the subchromatophore particle preparations rapidly destroys the special bacteriochlorophyll environment and the absorption maximum of "soluble" bacteriochlorophyll appears near 780 nm. These results suggest that the protein plays the major role in maintenance of the special environment for the light-harvesting bacteriochlorophyll in chromatophores.

The physical properties of these subchromatophore particles and their chemical compositions are consistent with a layered asymmetric structure of the chromatophore membrane. The light-harvesting bacteriochlorophyll is contained in a sheet of bacteriochlorophyll protein aggregate (light-harvesting component) and particles containing the reaction center bacteriochlorophyll with the other electron transfer compounds (reaction center complex) are situated at regular intervals on the surface of this aggregate. The non-ionic detergent Triton X-100 fractionates this membrane by removing the lipid and dissociating the reaction center complex particles from the large light-harvesting component particles. After removing the reaction centers and lipids, the light-harvesting component dissociates into small bacteriochlorophyll proteins in the presence of the anionic detergent sodium dodecyl sulfate and reaggregation in an apparently similar manner occurs after the detergent is removed. The average composition of the chromatophore membrane system can be accounted for by a reaction center complex containing the reaction center P870 with other compounds of the cyclic electron transfer system and a complement of light-harvesting bacteriochlorophyll in an aggregate of the sodium dodecyl sulfate fragment particles.

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