Some Structural and Photochemical Properties of *Rhodopseudomonas palustris* Subchromatophore Particles Obtained by Treatment with Triton X-100*

Augusto Garcia, Leo P. Vernon, Bacon Ke, and Hilton Mollenhauer

ABSTRACT: Treatment of *Rhodopseudomonas palustris* chromatophores with 0.6% Triton X-100 causes a significant change in the fluorescence spectrum from an emission peak at 895 mμ and a shoulder at 875 mμ to a more symmetrical peak at 867 mμ. This indicates the spatial arrangement of the three bacteriochlorophyll (Bchl) forms has been changed so that energy transfer from the 857-mμ form to the 873-mμ form is prevented. Treatment with 4% Triton produces two fragments separable by sucrose density gradient centrifugation. The heavy fraction is composed of particles which form linear aggregates which are 65–80 Å in thickness and which contain the photochemical apparatus of the chromatophore.

This fraction contains the long-wavelength Bchl form which absorbs at 873 mμ, as well as some of the shorter wavelength Bchl absorbing at 802 mμ. This fraction shows a light-induced bleaching (oxidation) of a portion of the 873-mμ Bchl, which represents the reaction center Bchl. Coupled to this is a photoreduction of endogenous ubiquinone. The light fragment produced by Triton shows no definite structure by electron microscopy, contains the 802- and 857-mμ forms of Bchl, and is devoid of the photochemical activities observed for the particulate fraction. It is concluded that the original photosynthetic membrane system contains the 802 and 857 forms of Bchl in the membrane matrix, that these Bchl molecules serve to harvest light energy, and that distributed on this membrane are the small, photochemically competent particles which initiate the photochemical reactions peculiar to bacterial photosynthesis.

The photosynthetic bacteria which contain bacteriochlorophyll (Bchl)[1] contain either exclusively or predominantly one Bchl, either Bchl a or Bchl b. *In vivo* the Bchl exhibits an absorption band in the orange and a series of bands in the near-infrared region. Although the exact location of the absorption maxima of the three Bchl a forms differs slightly in different bacteria, they are referred to as B800, B850, and B880. It is generally held that the different absorption properties of the Bchl molecules in the near-infrared region reflect different complexes of the Bchl with proteins (lipoproteins), and the study of Bril (1964) on the effects of detergents on the Bchl composition and fluorescence of chromatophores and derived fragments indicate that these Bchl–protein complexes can be separated, at least in part. Triton X-100, a nonionic detergent, was used (0.5%) by Bril (1964) to fractionate *Rhodopseudomonas spheroides* chromatophores into two fractions, one of which was enriched in the Bchl form absorbing at 870 mμ (B870) while the other contained the shorter wavelength forms of Bchl.

Garcia *et al.* (1966a,b) studied the fragments obtained from *Rhodospirillum rubrum* and *Chromatium* through the action of Triton X-100. Using higher concentration of the detergent (4%) two fragments were produced from each bacterium. The derived fragments were clearly separated by sucrose density gradient centrifugation, and called the light (L) and heavy (H) fragments. With *Chromatium* there was a clear separation of the three Bchl forms between the two fragments. The H fraction was particulate in nature, contained the B880 and some B800, and contained the reaction center Bchl, known as P890. The L fraction, on the other hand, was nonparticulate, contained the B850 and most of the B800, but was photochemically inactive. Somewhat different results were obtained with *R. rubrum*, in which case the L fraction was the particulate fraction, there was no separation of the Bchl forms (B880 and B800) between the two fragments, and photochemical activity was found in each fraction to the extent that each showed the presence of reaction center Bchl P890 and the light-induced electron spin resonance signal. This investigation concerns the fragments produced from *Rhodospeudomonas palustris* in this manner and considers the chemical and photochemical properties of these derived fragments.

Methods

R. Palustris cells were grown photosynthetically using the medium described by Cohen-Bazire *et al.*

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[1] Abbreviation used: Bchl, bacteriochlorophyll.

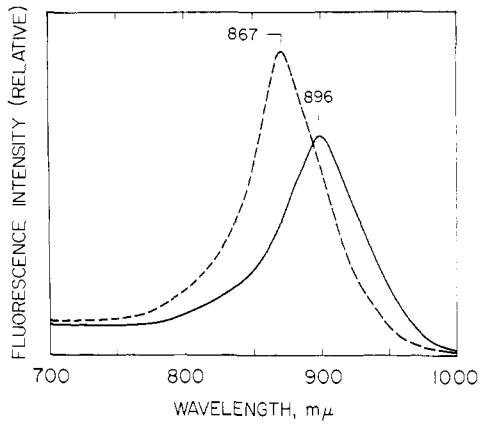

FIGURE 1: Fluorescence spectrum of *R. palustris* chromatophores (solid curve) suspended either in 0.05 M Tris-HCl buffer (pH 7.5) or in the same buffer with 0.6% Triton X-100 present. The spectrum of the treated chromatophores (dashed curve) was obtained after a 15–30-min incubation period with the detergent. The fluorescence was excited by light between 360 and 600 mμ obtained with the aid of sharp cut-off filter (Corning 9782). The Bchl concentration was 2 μM in both cases.

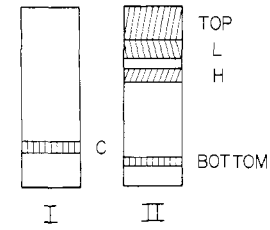

FIGURE 2: Sketch showing the distribution of untreated chromatophores (I) and the fragments produced with 4% Triton X-100 (II) after sedimentation through a discontinuous gradient of sucrose (see Methods section). During the incubation with Triton, the ratio of Bchl to Triton was between 3.5 and 5 mg of Bchl per 100 mg of Triton.

(1957), supplemented with 1% yeast extract and 1% peptone under a light intensity (tungsten lamp) of 500 ft candles. To prepare the chromatophore fraction, washed cells were suspended in 0.01 M Tris-HCl (pH 7.5) buffer and sonicated in a Raytheon sonic oscillator for 2 min at full power. The fraction that sedimented between 15,000 and 115,000*g* was used as the chromatophore fraction. This preparation was resuspended in the same buffer and applied to the top of a 15-ml layer of a 60% (w/v) CsCl solution in a centrifuge tube which was centrifuged for 60 min at 100,000*g*. The pigmented fraction remained on the surface of the CsCl solution and a colorless protein residue was detected in the bottom of the tube. This procedure greatly reduced the ratio of protein to Bchl in these preparations.

Chromatophores were treated with 4% Triton X-100 for 1 hr at 0°. The suspension of treated chromatophores was then applied to centrifuge tubes containing a discontinuous sucrose gradient consisting of three different layers (57, 24, and 14%) of sucrose dissolved in 0.01 M Tris (pH 7.7). The tubes were centrifuged for 12 hr at 110,000*g* in the 30 Rotor of a Spinco Model L ultracentrifuge. The light-induced absorbance changes were measured in the flash spectrophotometer previously described (Ke *et al.*, 1964). For measurements in the ultraviolet region the arrangement described by Ke *et al.* (1968) was used. The electron micrographs were taken with a Philips 200 electron microscope.

Bchl was determined after extraction of the material with a mixture of acetone–methanol (7:2, v/v), using an extinction coefficient of 75 mM^{-1} cm^{-1} at 772 mμ (Clayton, 1963). The fluorescence measurements and irradiation of the different fractions were made using the apparatus described by Mayne (1965). Absorption spectra were measured in a Cary 14 recording spectrophotometer. For protein determination a small aliquot of the sample was suspended in a mixture of acetone–methanol (7:2, v/v) and the suspension was centrifuged at low speed. The precipitate was washed twice with acetone–methanol, dried under reduced pressure, and redissolved in a 0.5 M solution of NaOH. An aliquot of this solution was used to determine protein concentration by the method of Lowry *et al.* (1961).

For the quinone determination, 1 ml of a nondiluted fraction was treated with 8 ml of a mixture of acetone–methanol (6:4, v/v). The resulting suspension was extracted twice with 8-ml portions of petroleum ether (bp 30–60°). The petroleum ether extracts were washed once with 8 ml of 95% methanol in water, once with distilled water, then dried over $CaCl_2$, and evaporated under reduced pressure. The dried residue was dissolved in isooctane and purified by means of thin-layer chromatography, using silica gel as the supporting phase and chloroform as developing solvent. The plates were sprayed with a solution of reduced methylene blue in sulfuric acid, which gave blue spots on the plates for the different quinone compounds. For a quantitative determination of the quinone, the reduced minus oxidized spectrum of the thin-layer chromatography purified material was determined after extraction into ethanol using a differential extinction coefficient of 12 mM^{-1} cm^{-1} at 275 mμ (Crane and Dilley, 1963).

Results

Fluorescence Measurements. The emission spectra of *R. Palustris* chromatophores suspended in buffer or in buffer plus 0.6% Triton X-100 are shown in Figure 1. The spectrum for the chromatophores alone shows an asymmetrical band with a peak at 896 mμ and a shoulder at 875 mμ. The presence of the shoulder indicates there is not complete transfer of excitation energy between the B857 and B873, so that some fluorescence is obtained from the B857. Upon addition

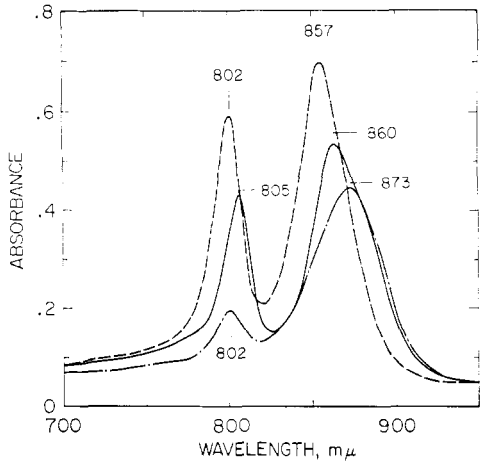

FIGURE 3: Absorption spectra of *R. palustris* chromatophores (solid curve) and derived fragments (dashed and dot curve for H; dashed curve for L) in the near-infrared region. The H and L fragments were obtained by density gradient centrifugation and were diluted with 0.05 M Tris buffer (pH 7.5) prior to measurement of the absorption spectrum. The Bchl concentrations in 3 ml were: chromatophores, 0.008 μmole; H, 0.003 μmole; L, 0.011 μmole. The optical path length was 1 cm.

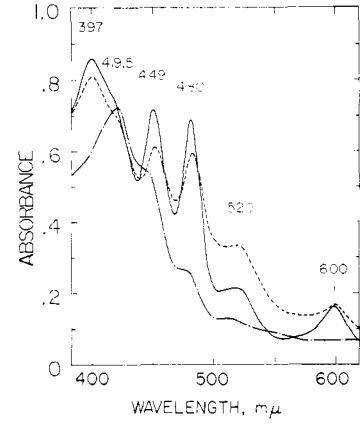

FIGURE 4: Absorption spectra of *R. palustris* chromatophores (solid curve), the H and L fragments (dashed curve), and the top fraction (dashed-dotted curve). The Bchl concentration in 3 ml of chromatophore suspension was 0.008 μmole. The Bchl concentration for H and L (dashed curve) was not measured. Since identical spectra were obtained for both the H and L fractions in this wavelength region, only one spectrum is given.

of the detergent, the emission spectrum changes to show a single, more symmetrical band with a peak at 867 mμ. The latter band is skewed toward longer wavelengths, however, which indicates there is some contribution to the fluorescence spectrum by the longer wavelength Bchl, but the main fluorescence comes from the B857. Thus, the spatial organization has been altered so that energy transfer between B857 and B873 is prevented. Similar results were obtained by Bril (1964) with *Chromatium*. Since the bulk of the fluorescence is from the B857, there is efficient transfer of excitation energy between the B802 and B857, which indicates that their relationship is not affected by the detergent.

An evaluation of the different mechanisms for energy transfer (Rabinowitch, 1962; Bay and Pearlstein, 1963) shows that transfer of excitation energy by inductive resonance is the main if not the only mechanism in photosynthetic tissues. This mechanism allows transfer of energy over distances of 100 Å, provided certain conditions are satisfied. The effect observed for Triton X-100 indicates that at a concentration of 0.6% the B873 is effectively removed over 100 Å from the other two Bchl forms, whose relationship to each other is not sufficiently altered to affect energy transfer between these moieties.

Chromatophore Fragments. Treatment of *R. palustris* chromatophores with 4% Triton X-100 for 1 hr at 0° causes a rupture of the photosynthetic apparatus. The fragments so produced may be separated by centrifugation through a discontinuous gradient of sucrose (see Methods section). Figure 2 portrays the appearance of the centrifuge tube after the separation is complete. Part I shows the position of untreated chromatophores in the centrifuge tube, while part II shows the location of the derived fragments, which are called heavy (H) and light (L) fractions according to their position in the tube. The small amount of material which sediments toward the bottom of the tube is unchanged chromatophore material, since reextraction of this material allows the formation of additional H and L material. Further extraction of the H fraction with Triton X-100 liberates small additional amounts of the L fragment. The separation obtained by this procedure resembles that reported earlier for *Chromatium* chromatophores (Garcia *et al.*, 1966a). There is some reddish-yellow material which accumulates at the top of the tube. Examination of the spectrum of this band shows that it contains carotenoids, which have been separated from the main Bchl complexes by this treatment.

The absorption spectra of *R. palustris* chromatophores as well as for the derived fragments are given in Figures 3 and 4. The greatest difference is seen in Figure 3, which presents the absorption spectra for the near-infrared region. The chromatophore fraction shows absorption peaks at 805 and 860 mμ, with a shoulder at approximately 880 mμ. The H fragment obtained by Triton treatment shows a major peak at 873 mμ, with a smaller band at 802 mμ, showing clearly that the long-wavelength Bchl form is concentrated in this fragment. Therefore, the two shorter wavelength forms are found in the L fraction, with peaks at 857 and 802 mμ. The small amount of B802 appearing in the H fragment is presumably the special B800 material located close to the reaction center Bchl,

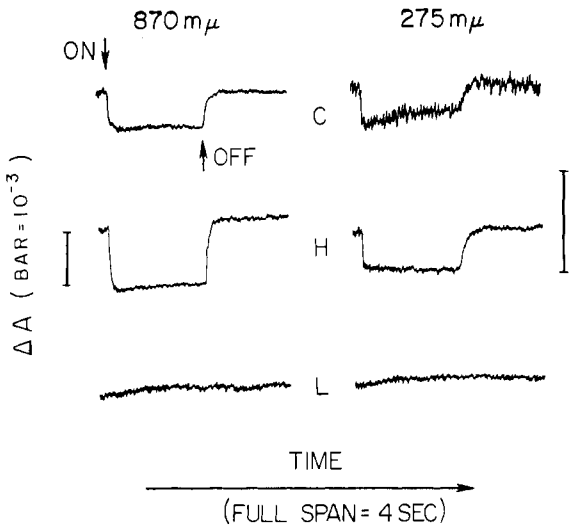

FIGURE 5: Light-induced absorbance changes corresponding to P870 photooxidation and the coupled reduction of endogenous ubiquinone at 275 mμ for the H and L fractions as well as chromatophores (C). Actinic light at 875 mμ (interference filter) was used for measuring the quinone changes and a broad-band blue filter was used when P870 was determined. The Bchl concentrations in 3.0 ml were: 0.013 (H), 0.01 (L), and 0.017 μmole (chromatophores). The intensities of both actinic lights were 10^5 ergs/cm^2 sec.

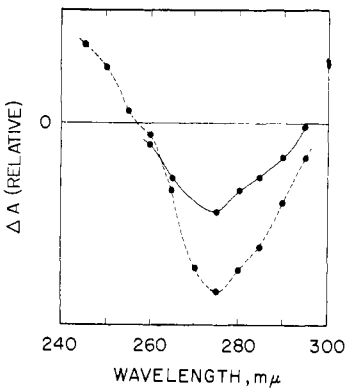

FIGURE 6: Light minus dark difference spectrum in the ultraviolet region showing the photoreduction of ubiquinone by the H fraction (dashed curve) and chromatophores (solid curve) of *R. palustris*. The conditions were those given for Figure 5 except for the Bchl concentrations, which were (in 3.0 ml): 0.008 (H) and 0.012 μmole (chromatophore).

which has recently been described by Clayton (1966a,b). The bulk of the B802 is light-harvesting Bchl, and as expected, it appears along with the B857 on the light fragment.

Perusal of Figure 4 shows that the original chromatophores and the H and L fragments have similar absorption properties in the visible region, showing the 600- and 397-mμ peaks of Bchl and the usual absorption of the carotenoids in the 400–500-mμ region. Also shown is the spectrum of the top fraction, which contains carotenoid but no Bchl.

Reaction Centers. Illumination of chromatophores of photosynthetic bacteria causes a bleaching of a small fraction of the long-wavelength from of Bchl. This change is mimicked by treatment with ferricyanide, which led Clayton (1962a) to propose that this Bchl is oxidized in the light and serves at the site of the primary photoreaction; he called it the reaction center Bchl. This specialized Bchl is also designated by the wavelength of its absorption change, such as P890 or P870 (Clayton, 1966b). The earlier investigation on *Chromatium* (Garcia *et al.*, 1966a) showed that the reaction center was localized in the H fraction, which also contained the long-wavelength Bchl. A similar situation is observed in *R. palustris*. Figure 5 shows the light-induced absorption changes at 870 mμ which correspond to the light-induced oxidation of this reaction center Bchl. The data show that this change is observed in the chromatophores and the H fraction (the signal is actually accentuated), but not at all in the L fragment. This is expected, since the L fragment contains none of the B873.

If the P870 is photooxidized in a primary event, some other molecule must become reduced. There is growing evidence that the compound serving this role is ubiquinone. Clayton (1962b) showed that with *R. spheroides*, light caused an absorbancy change in the ultraviolet region which indicated a photoreduction of a quinone. Bales and Vernon (1963) later reported that the photooxidation of a reduced indophenol dye by *R. rubrum* chromatophores was accompanied by a reduction of endogenous quinone. Ke *et al.* (1968) have studied in detail the photoreduction of endogenous ubiquinone in *Chromatium* H particles, and show that the photoreduction of ubiquinone is coupled to the photooxidation of reaction center Bchl. Figure 5 shows that a similar reaction takes place with *R. palustris*. The quinone reaction was followed at 275 mμ, and the data show the presence of the quinone photoreduction in chromatophores and the H particle. Again the L particle is devoid of this activity.

Figure 6 shows the light minus dark difference spectrum in the ultraviolet region for *R. palustris* chromatophores and H fraction. In both cases a minimum in the difference spectrum is observed at 275 mμ. Isosbestic points are observed at 247 and about 290 mμ for the H fraction. This difference spectrum closely resembles the light minus dark difference spectrum for ubiquinone in *Chromatium* fragments (Ke *et al.*, 1968). In examining the quinone content of the chromatophores it was noted that whereas a prominent band was noted in the ubiquinone region, another major spot appeared in the region expected for vitamin K. Further work is underway on this unknown quinone. The data of Figure 6, however, show that the main photoreduction involves the endogenous ubiquinone.

TABLE I: Distribution of Bacteriochlorophyll and Ubiquinone between the H and L Fractions Obtained through the Action of Triton X-100.[a]

Fraction	μmoles of Bchl/mg of Protein	μmoles of Bchl/μmoles of Quinone
Chromatophore	0.018–0.02	8.7
L fraction	0.02–0.03	5.4
H fraction	0.12	6.7

[a] See the Method section for details concerning the quantitative methods used.

Table I shows the distribution of Bchl and ubiquinone among the various fractions. It is seen that the various fractions contain a fairly constant ratio of ubiquinone to Bchl. Thus, the inactivity of the L fraction for ubiquinone photoreduction is not due to the lack of the quinone, but rather is due to the lack of the reaction center Bchl. The data of Table I also show that Bchl is more concentrated in the H fraction, whereas the chromatophore and L fraction have about the same ratio of Bchl to protein. In terms of total Bchl in the original chromatophore, the H fraction accounts for approximately 50–60% while the L fraction contains approximately 40%.

Structure. The fractions obtained with Triton have been examined in the electron microscope, using the technique of negative staining. Figure 7 (7) shows a chromatophore preparation, showing the presence of the typical small disk-shaped chromatophore as well as a larger structure which represents a portion of the photosynthetic apparatus as it occurs in the cell. Thin sections of whole *R. palustris* cells show that the photosynthetic system consists of lamellar arrangement of the membranes which contain the Bchl (Cohen-Bazire and Sistrom, 1966). Figure 7 (7) shows that the process of sonication breaks down this structure to produce the more typical small chromatophore structure, but that some of the lamellar arrangement also appears in this fraction. Only occasionally is there any indication of any substructure on the chromatophore membrane.

Figure 7 (8) shows an electron micrograph of the H fraction. The main feature of this micrograph is the long strands of material, which are completely different from the original chromatophore structure. Close examination shows that the strands are composed of individual units which have joined together in a linear array. The investigation on *Chromatium* (Garcia *et al.* 1966a) showed that the H particle, which corresponds to the *R. palustris* H particle, consists of small particles which are fairly spherical. In the present case the Triton treatment has liberated a small particle from the surface of the *R. palustris* chromatophore, but the particles tend to spontaneously condense in the linear formation shown in Figure 7 (8). Examination of the L fraction showed material which had no visible structure to it. Rather, this fraction appeared poorly defined, resembling what one would see for a solution of protein or lipid.

Discussion

The studies of Bril (1964) on the fluorescence properties of detergent-treated chromatophores from purple bacteria show that low concentration of detergents disrupt the energy flow between the B850 and B880 forms of Bchl. The successful fractionation of these chromatophores using Triton X-100 (Garcia *et al.*, 1966a,b) has confirmed the belief that a change in the spatial arrangement of the different Bchl–protein complexes will produce a drastic change in the fluorescence properties of the treated chromatophores. The data presented in this paper show that with *R. palustris* also, a change in the internal structure of the chromatophore fraction is brought about by Triton X-100. The separation of different fractions with different physical and photochemical properties indicates that the bulk Bchl of this photosynthetic bacterium is contained in at least two distinct environments.

The nature and the function of the L fraction seem to be, as was postulated for *Chromatium* (Garcia *et al.*, 1966a), that of an energy collecting system, lacking the necessary components to employ this energy in photochemical work. On the other hand, the H fraction has several properties which identify it with the active part of the photochemical apparatus. These are the following. (1) It contains the long-wavelength form of Bchl (B870) whose absorption coincides with the absorption maximum of the specialized Bchl in the reaction center. The small absorption band detectable at 802 mμ does not seem to be an "impurity" coming from the L fraction, since repeated extractions of the H fraction with additional amounts of Triton X-100 do not seem to greatly affect the ratio B870:B800. Furthermore, it has been proposed by Clayton (1966a,b) and by Olson and Clayton (1966), that in *R. spheroides*, a pigment absorbing at 800 mμ (P800), appears to belong to a pigment complex that is associated with the usual P870 Bchl form of the photosynthetic reaction center, and that in *Rhodopseudomonas* species NHCT 133, a special form of Bchl b, the short waveform P830, is a special form of Bchl b in close association with the reaction center pigment P985. In the latter case, the P830 pigment is more effective in sensitizing cytochrome oxidation and P985 bleaching than in exciting fluorescence of Bchl b. Accordingly, the B800 form remaining in the H fraction of *R. palustris*, represents a small amount of B800 which is associated with the reaction center. (2) An operating reaction center associated with the H fraction is detected by the light-induced absorbance changes at 870 mμ. This change is due to a reversible photooxidation of a small fraction of the P870 form of Bchl. (3) Concomitant with this absorbance change there is another absorbance change centered at 275 mμ. Ke *et al.* (1968) have shown

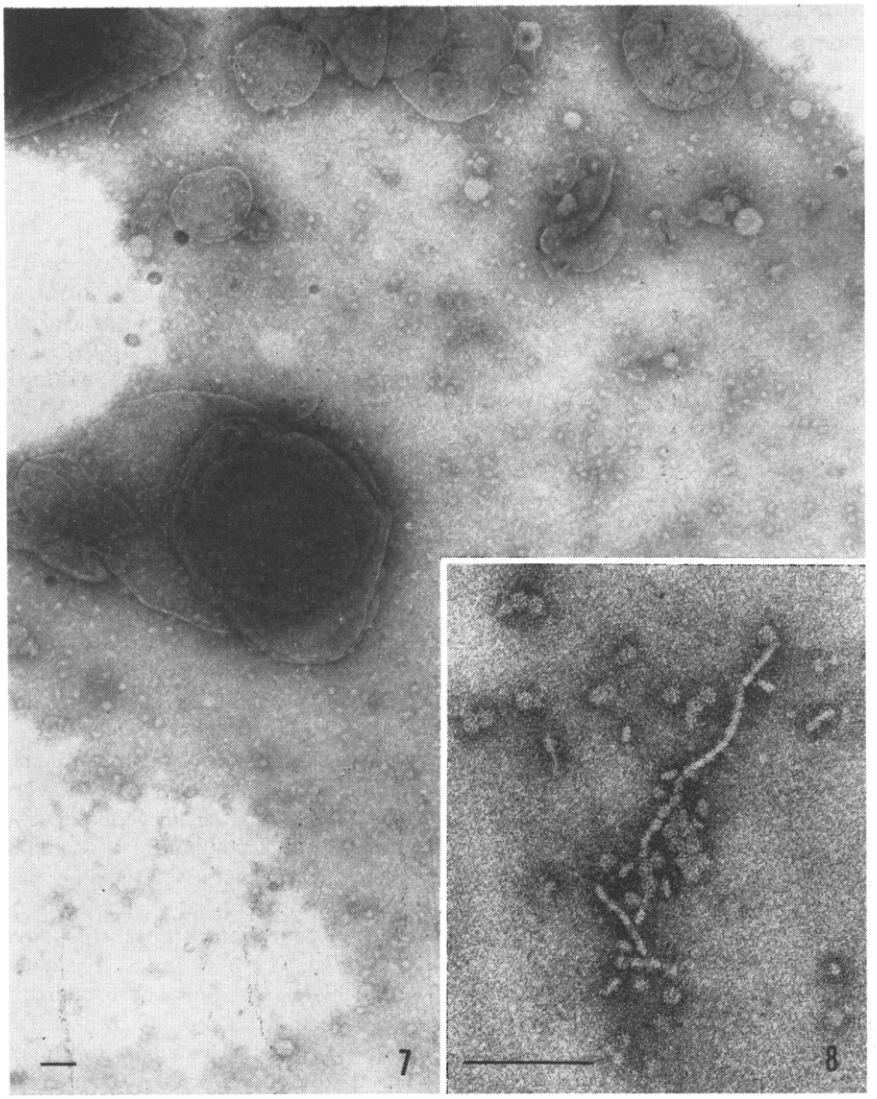

FIGURE 7: Electron micrographs. (7) Electron micrograph of a chromatophore preparation of *R. palustris*. Negative staining was done with phosphotungstic acid at pH 5.9 in the presence of 5×10^{-3} M $MgCl_2$. The bar represents 1000 Å. (8) Electron micrograph of the H fraction of *R. palustris*. Negative staining with phosphotungstic acid at pH 5.9 with 5×10^{-3} M $MgCl_2$ was used to prepare the samples. The particles formed linear aggregates which varied from 65 to 80 Å in thickness. The bar represents 1000 Å.

that in *Chromatium* chromatophores and in the H fraction derived from it by Triton treatment, the change at 275 mμ corresponds to the photoreduction of endogenous ubiquinone. The chemical and kinetic characteristics of this absorbance change are similar to the corresponding chemical and kinetic characteristics of the reaction center Bchl. This is consistent with the view expressed before, that the endogenous ubiquinone functions as the initial electron acceptor in the primary photochemical act in these organisms. Although in the case of *R. palustris* no detailed study of the relationship between the absorbance change at 275 and 870 mμ has been carried out, it is likely that the 275-mμ change represents the change in redox state of the endogenous ubiquinone coupled to reaction center Bchl, since the 275- and 870-mμ changes always occur together. (4) A comparison of the structural properties of the L and H fractions shows that the L fraction does not have any definite form and appears to consist of soluble material. The H fraction, on the other hand, is a "particulate fraction" in which the small particles are aggregated into linear arrays. It is difficult to see much detail in the substructure of these membranes in the original chromatophore to relate to the H fragment. However, the important point to emphasize is that the H and the L fractions arise from two different environments with very different structural properties.

The L fraction, whose function is postulated to be one of gathering light energy, does not reveal any

regularity in its structure, as would be expected from its possible function. On the other hand, the H fraction containing the photochemically active part of the chromatophore vesicles has a more complex and definite structure. According to the results presented above, the photochemical apparatus can be visualized as consisting of a basic membrane, probably containing in addition to the usual phospholipids, proteins, etc., some of the enzymes involved in the "dark" processes of photosynthetic electron transport. The small particles, containing mainly the photochemical machinery, are imbedded in this basic membrane in such a way as to form a very efficient association with the enzymes operating in the dark process. Sonication of the intact cells will comminute the membrane and produce the so-called chromatophore fraction. As shown in Figure 7, this fraction consists of fragments of the membrane which adopt the most probable shape according to their physicochemical properties. Upon addition of the detergent, the basic membrane structure would be solubilized along with its associated pigment, releasing the small particles (H fraction). In this regard it resembles the other purple photosynthetic bacteria examined to date. Of the bacteria tested, however, *R. palustris* most nearly resembles *Chromatium*. The properties of the two fragments so produced are very similar for these two bacteria.

Acknowledgments

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